COMBINATORIAL METHODS FOR INDUCING CANCER CELL DEATH

SPECIFICATION

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1. INTRODUCTION

The present invention relates to methods and compositions for inhibiting proliferation and inducing cell death in a population of cancer cells by (i) increasing the amount of the differentiation associated protein *MDA*-7, and (ii) decreasing *RAS* activity within the population. It is based, at least in part, on the discovery that decreasing expression of a mutated, activated K-*ras* gene, together with introducing an expressible *mda*-7 gene, in pancreatic cancer cells had a synergistic growth-inhibitory and anti-survival effect, and abolished tumorigenicity of the cells in athymic nude mice. The invention provides for methods of treating patients suffering from pancreatic cancer or other malignancies.

2. BACKGROUND OF THE INVENTION

2.1. PANCREATIC CANCER

Pancreatic cancer is the eighth most frequent type of solid tumor arising worldwide, but, as a consequence of the current lack of effective therapy, it is the fourth most frequent cause of cancer death (Gunzburg and Salmons, 2001, Trends Mol. Med. 7(1):30-37). It is estimated that 29,200 cases will be diagnosed in the United States in 2001, and 28,900 of these patients are expected to die (Cancer Facts and Figures, 2001. Atlanta, GA; American Cancer Society, 2001). Long-term survival for patients with organ-confined disease is only 20 percent, and in the majority of cases, in which the disease, when diagnosed, has already spread past the pancreas, survival is only 4 percent (Hilgers and Kern, 1999, Genes, Chromosomes & Cancer 26:1-12; Regine et al., 1998, Front. Biosci. 3:E186-E192; Blaszkowsky, 1998, Front. Biosci. 3:E214-E225; Lorenz et al., 2000, Eur. J. Cancer 36:957-965; Rosenberg, 2000, Drugs 59:1071-1089).

The poor prognosis associated with pancreatic cancer has been attributed to a number of factors. These include (1) the anatomic location of the pancreas and lack of specific early symptoms make early diagnosis difficult; (2) the tumor spreads rapidly to surrounding vital organs; (3) even small tumors tend to metastasize; and (4) the cancer generally responds poorly to standard therapeutic measures (Aoki et al., 1995, Cancer Res. 55:3810-3816, citing Yamaguchi et al., 1989, Jpn. J. Clin. Oncol. 19:14-22; Warshaw and Castillo, 1992, N. Engl. J. Med. 326:455-465; Cohn, 1989, Int. J. Pancreatol. 7:1-11; Ozaki et al., 1992, Int. J. Pancreatol. 12:5-9; Arbuck, 1990, Int. J. Pancreatol. 7:209-222).

The lethality of pancreatic cancer has warranted extreme therapeutic measures. A recent study suggests that multimodal therapy, combining pancreaticoduodenectomy with postoperative adjuvant chemotherapy (using fluorouracil) and external beam radiation therapy maximizes local tumor control and improves the length of survival (Evans et al., 2001, Oncology (Huntingt) 15(6):727-737). When the tumor has been unresectable, combination chemotherapy with gemcitabine and docetaxel has achieved modest success in decreasing tumor mass and or/serum tumor markers (Sherman and Fine, 2001, Oncology 60(4):316-321).

2.2. THE MOLECULAR BIOLOGY OF PANCREATIC CANCER

To better treat this aggressive tumor, scientists are attempting to achieve an understanding of pancreatic cancer at the molecular level. A number of molecules and pathways have been implicated as either playing an etiologic role or creating therapeutic opportunities, including: fibroblast growth factors, as modulators of the E-cadherin/catenin system (Hariry et al., 2001, Br. J. Cancer 84(12):1656-1663); the 26S proteasome (Shah et al., 2001, J. Cell. Biochem. 82(1):110-122); cyclic adenosine monophosphate (cAMP), acting with other second messengers to mediate signals from tumoral growth hormone releasing hormone receptors (Rekasi et al., 2001, Peptides 22(6):879-886); the CD95 (FAS-Apo-1) apoptosis pathway, which is reported to be potentially functional, but blocked by an unknown protein in pancreatic cancer cells (Glazyrin et al., 2001, Pancreas 22(4):357-365); P38 MAP kinase, as a negative regulator of MEK/ERK-mediated proliferation (Ding and Adrian, 2001, Biochem. Biophys. Res. Commun. 282(2):447-453), and transforming growth factor beta 1 ("TGF-β1"; Giehl et al., 2000, Oncogene 19(39):4531-4541;

Hashimoto et al., 2001, Pancreas 22(4):341-347; Ellenrieder et al., 2001, Int. J. Cancer 93(2):204-211).

In pancreatic tumors, a high incidence of overexpression of TGF-βs and their receptors, as well as activating mutations of the K-*ras* oncogene, have been reported, suggesting that "interactions of the *RAS* cascade and the TGF-β pathway may play an important role in pancreatic carcinogenesis" (Ellenrieder et al., 2001, Cancer Res. 61:4222-4228). In experiments to test this hypothesis, TGF-β1 treatment of responsive pancreatic cancer cells having activating K-*ras* mutations resulted in an epithelial-mesenchymal transdifferentiation and a more invasive phenotype which could be reduced or abolished by pretreatment with a MEK1 inhibitor (*Id.*).

Multiple subsets of genes have been observed to undergo genetic change, either activation or inactivation, during tumor development and progression (Hilgers and Kern, 1999, Genes, Chromosomes & Cancer 26:1-12; Perugini et al., 1998, Crit. Rev. Eukaryotic Gene Express. 8:377-393; Friess et al., 1999, Dig. Surg. 16:281-290). Frequent genetic modification in pancreatic carcinomas include activation of the K-ras oncogene (85 to 95 percent) and inactivation of the p16/RB1 (>90 percent), p53 (75 percent) and DPC4 (55 percent) tumor suppressor genes (*Id.*). These findings highlight the complexity of pancreatic cancer and may provide a partial explanation for the aggressiveness and inherent resistance of this neoplasm to conventional therapies such as chemotherapy and radiation (Regine et al., 1998, Front. Biosci. 3:E186-E192; Blaszkowsky, 1998, Front. Biosci. 3:E214-E225; Lorenz et al., 2000, Eur. J. Cancer 36:957-965; Rosenberg, 2000, Drugs 59:1071-1089).

2.3. *RAS* PROTEINS

Of the genetic changes that occur in pancreatic cancers, mutations in the K-*ras* gene, predominantly in codon 12, are the most frequent (Hilgers and Kern, 1999, Genes, Chromosomes & Cancer 26:1-12; Almoguera et al., 1988, Cell 53:549-554; Longnecker and Terhune, 1998, Pancreas 17:323-324). *RAS* proteins constitute a family of eukaryotic cellular proteins that act to assist in the transmission of information from the outside of the cell to the inside, resulting in changes in the fundamental properties of the cell, such as levels of gene transcription and expression, growth status and differentiation state (Campbell et al., 1998, Oncogene 17:1395-1341; Kolch, 2000, Biochem. J. 351:289-305). As such, *RAS* proteins can play an important role in the transformation of a cell to a cancer cell, a process referred to as "oncogenesis". The *ras* family contains three functional so-called "oncogenes", namely H-*ras*, K-*ras*, and N-*ras*, which encode highly similar proteins with molecular weights of 21,000 daltons (Reuther and Der, 2000, Curr. Opin. Cell Biol. 12:157-165; note that the proteins and genes of this family are referred to herein by upper case and lower case italics, respectively, for consistency within this document (see below) but contrary to popular convention).

Functionally, a *RAS* protein alternates between two forms in the cell. When unattached to the cell membrane and bound to the compound GDP (guanine diphosphate), the *RAS* protein is in its biologically inactive state. For *RAS* to become active, several events must occur. First, the protein must be chemically modified by a process called farnesylation. This modification attaches a fatty acid side chain onto the *RAS* protein, enhancing its ability to associate with the lipid-rich inner cell membrane. Once anchored to the cell membrane, *RAS* can then interact with several other proteins to complete its activation. These include membrane-spanning protein receptors that bind informational molecules that are presented on the outside of the cell membrane, and a variety of accessory molecules that mediate the interaction between *RAS* and the receptor protein. These latter molecules, so-called exchange and adapter proteins, also assist in the release of GDP from the *RAS* protein and the binding of GTP (guanine triphosphate), which is the final step in activation.

Activation of the wild-type *RAS* proteins is a reversible process. *RAS* itself is also a GTPase, that is, it hydrolyzes GTP to form GDP. The rate of this conversion, which is greatly enhanced by other cellular proteins known as GTPase-

activating proteins (GAPs), is the key factor that determines how long the *RAS*-mediated signal persists in the cell. Once activated, *RAS* triggers a cascade of signals that are conveyed from the cell membrane into the nucleus of the cell. A diagram depicting a simplified version of the *RAS* pathway is presented in FIGURE 1. These signals are mediated by a series of kinases, enzymes that catalyze the phosphorylation of cellular proteins. Ultimately, this pathway results in the activation of nuclear proteins called transcription factors, which act to increase the rate of transcription of specific genes within the cell. When activated, K- *RAS* can signal into the cytosol via multiple downstream signaling pathways such as the classical MAPK pathway, the phosphatidylinositol ("PI3") kinase pathway, and the JNK pathway, to induce a plethora of cellular changes, including enhanced proliferation and cell survival (Dent et al., 1992, Science 257:1404-1407; Gire et al., 2000, Oncogene 19:2269-2276; Almeida et al., 2000, J. Cell Biol. 149:741-754).

Many of the genes whose transcription are upregulated by *RAS* activation are involved in the control of cell cycling, and thus persistent activation of *RAS*, which can be caused by mutations in the *ras* gene which impair the protein's GTPase activity, can lead to abnormalities in cellular proliferation. In fact, this mechanism has been implicated in the development of a wide variety of human cancers. A common occurrence in pancreatic and other cancers involves point mutations of K-*ras*, which may involve codon 12 (a common mutation in pancreatic cancer cells) and codons 13 and 61 (more common in other cancers) (Hilgers and Kern, 1999, Genes, Chromosomes & Cancer 26:1-12; Almoguera et al., 1988, Cell 53:549-554; Longnecker and Terhune, 1998, Pancreas 17:323-324). For example, Noda et al. (2001, Oncol. Rep. 8(4):889-92) report mutations at codons 12, 13 and 61 of K-*ras* in non-small cell lung cancer tumors. Demunter et al. (2001, Cancer Res. 61:4916-4922) report a novel mutation in codon 18 of exon 1 of the N-*ras* gene which was found in 15 percent of primary malignant melanoma tumors studied but not in any metastatic melanoma. Other cancers associated with point mutations of *ras* include gallbladder carcinoma (Kim et al., 2001, Cancer Lett. 169(1):59-68) and colon carcinoma (Clarke et al., 2001, Int. J. Colorectal Dis. 16(2):108-111).

In view of the association between *ras* mutation and cancer, a number of researchers have attempted to reverse oncogenesis in cells by selectively inhibiting the mutated *ras* gene. For example, interference in expression of K-*ras* using antisense RNA (Aoki et al., 1995, Cancer Res. 55:3810-3816; Aoki et al., 1997, Mol.

Carcinogen. 20:251-258) or with K-*ras* mutation-specific phosphorothioate oligodeoxynucleotides (Kita et al., 1999, Intl. J. Cancer 80:553-558) has been observed to inhibit the growth of pancreatic cancer cells containing K-*ras* mutations, but not pancreatic carcinoma cells containing a non-mutated wild-type K-*ras* gene. The dominant negative H-*ras* mutant, N116Y, was found to suppress pancreatic cancer cell growth *in vitro* and *in vivo*, including tumorigenesis and metastasis to the liver of nude mice (Shichionohe et al., 1996, J. Surg. Res. 142:63-71; Takeuchi et al., 2000, Gene Ther. 7:518-526). Although promising, these studies demonstrated that a single approach of inhibiting K-*ras* is not sufficient to completely eradicate pancreatic carcinoma cells (Aoki et al., 1995, Cancer Res. 55:3810-3816; Aoki et al., 1997, Mol. Carcinogen. 20:251-258; Kita et al., 1999, Intl. J. Cancer 80:553-558; Shichionohe et al., 1996, J. Surg. Res. 142:63-71).

2.4. *MDA-7*

A critical element of the present invention, the *mda*-7 gene (named as a melanoma differentiation associated gene), was previously found ineffective in modifying the malignant properties of pancreatic cancer cells. *Mda*-7 was identified by a subtractive hybridization technique using cDNA libraries prepared from actively proliferating melanoma cells and from melanoma cells which had been induced to terminally differentiate by treatment with recombinant human fibroblast interferon (IFN-β) and the protein kinase C activator mezerein (Jiang and Fisher, 1993, Mol. Cell. Different. 1:285-299; Jiang et al., 1995, Oncogene 11:2477-2486). *MDA*-7 has been characterized as a protein having 206 amino acids with a size of 23.8 kDa and a sequence as set forth in SEQ ID NO:1 (Genbank Accession Number U16261; Jiang et al., 1995, Oncogene 11:2477-2486).

When the *mda*-7 gene was introduced into a wide spectrum of human cancers, growth of cancer cells was inhibited (United States Patent No. 5,710,137 by Fisher, issued January 20, 1998; Jiang et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:9160-9165; Su et al., 1998, Proc. Natl. Acad. Sci. U.S.A. 95:14400-14405; Madireddi et al., 2000, Adv. Exptl. Med. Biol. 465:239-261). *MDA*-7 has been observed to suppress growth in cancer cells which either do not express, or which contain defects in, both retinoblastoma ("*rb*") and p53 tumor suppressor genes, indicating that *mda*-7 mediated growth inhibition does not depend on these elements (Jiang et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:9160-9165). In contrast to the

anti-proliferative effect on various cancer cells, no significant growth inhibitory effect was apparent when this gene was introduced into normal human fibroblast or epithelial cells (Jiang et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:9160-9165; Madireddi et al., 2000, Adv. Exptl. Med. Biol. 465:239-261; Saeki et al., 2000, Gene Ther. 7:2051-2057; Mhashilkar et al., 2001, Mol. Med. 7:271-282).

Despite its inhibitory effects on a variety of tumors, *mda-7* was not found to be effective against pancreatic carcinoma cells. The present invention is based on the discovery that although neither introduction of an *mda-7* gene nor inhibition of *RAS* have been observed to effectively control pancreatic cancer cell growth, their combination produces a synergistic effect, resulting in a dramatic suppression in cell growth and decrease in cell viability. The potency of *mda-7*/anti-*RAS* against a cancer as aggressive and rapidly lethal as pancreatic cancer is unexpected in view of the lack of effectiveness of either agent, used alone. In addition, the fact that *RAS* inhibition appears to be required for *MDA-7* to be effective is surprising in view of the fact that *MDA-7* has been observed to exert an anti-proliferative effect in the context of increased *RAS* activity; introduction of an *mda-7* gene had a strong anti-proliferative effect on rat embryo fibroblasts transformed with the H-*ras* oncogene (Jiang et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:9160-9165).

3. SUMMARY OF THE INVENTION

The present invention relates to methods and compositions for inhibiting proliferation and inducing cell death in a population of cancer cells by (i) increasing the amount of the differentiation associated protein, *MDA-7* and (ii) decreasing *RAS* activity within the population. *RAS* activity may be decreased by agents directed at *RAS* itself or at molecules upstream or downstream of *RAS* in the *RAS* pathway such as, for example, the epidermal growth factor receptor ("*EGFR*"), *RAF*, *MAPK* kinase, *MAPK* and PI3 kinase. In preferred embodiments, the methods include (i) increasing the amount of *MDA-7* protein and (ii) decreasing the expression of an activated *ras* gene in a cancer cell. These methods and compositions may be directed toward the treatment of subjects suffering from cancer, particularly pancreatic cancer.

The invention is based, at least in part, on the discovery that apoptosis of pancreatic cancer cells having activating mutations in K-*ras* could be induced by introducing an *MDA-7*-encoding nucleic acid and by inhibiting K-*RAS* expression. In

particular, it was found that introduction, into the pancreatic cancer cells, of antisense nucleic acids targeted at K-*ras*, together with a replication defective adenovirus carrying the *mda*-7 gene, not only induced apoptosis, but also inhibited malignant cell colony formation *in vitro* and tumor formation *in vivo* in nude mice.

Accordingly, the present invention provides for methods of inhibiting the proliferation and/or decreasing the viability of cancer cells, particularly pancreatic cancer cells, which carry an activated *ras* gene, and for therapeutic regimens which utilize such methods. Methods are provided for determining whether a particular cancer cell is likely to be responsive to the methods of the invention.

The present invention further provides for compositions which may be used to increase expression of *MDA*-7 and/or decrease *RAS* activity. In one specific, non-limiting embodiment, the present invention provides for an adenovirus vector comprising a sequence encoding *mda*-7 and a sequence encoding antisense *ras*, in expressible form.

In additional aspects of the invention, it has been discovered that the culture supernatant of cells expressing *MDA*-7 has an antiproliferative effect on pancreatic cancer cells when co-administered with antisense *ras* molecules. Accordingly, the present invention provides for methods for inhibiting cancer cell proliferation comprising exposing cancer cells to extracellular *MDA*-7.

4. DESCRIPTION OF THE FIGURES

FIGURE 1. Simplified diagram of the *RAS* pathway.

FIGURE 2A-C. Preparation of replication-defective adenovirus containing an *MDA*-7 encoding nucleic acid (*i.e.*, a *mda*-7 gene). (A) shows the pCMV/*mda*-7 plasmid; (B) shows recombination between pCMV/*mda*-7 and pJM17 in 293 cells; and (C) shows the product Ad.*mda*-7 virus.

FIGURE 3A-C. Preparation of replication-defective adenovirus containing an AS K-*ras* encoding nucleic acid . (A) shows the pCMV/K-*ras* AS plasmid; (B) shows recombination between pCMV/K-*ras* AS and pJM17 in 293 cells; and (C) shows the product Ad.K-*ras* AS virus.

FIGURE 4. Diagram showing the preparation of bipartite Ad.*mda*-7/K-*ras* AS. The figure is not drawn to scale, but terminal nucleotides are shown, based on the length of 3595 nucleotides for Ad serotype 5, from which this vector is derived. The ovals containing the letters "TP"represent the terminal protein

covalently bound to the native genome. The cytomegalovirus immediate early (CMV) promoter was used to drive the transcription of both *mda*-7 and K-*ras* AS from the bipartitie vector.

FIGURE 5. Synergistic inhibition of growth in mutated K-*ras* pancreatic carcinoma cells (pancreatic carcinoma cells containing a mutation in the K-*ras* gene) by the combination of Ad.*mda*-7 (a replication-defective adenovirus carrying the *mda*-7 gene in expressible form) and AS K-*ras* PS ODN (a phosphorothioate linked antisense *ras* oligonucleotide). Cells were treated with the indicated agents for three days and viable cell counts were determined by hemocytometer. AS PS ODN: 0.5 or 5.0 μM; Ad.*mda*-7: 100 plaque-forming units ("pfu")/cell; MM PS ODN (mismatched oligonucleotide): 5.0 μM. Results are the average of four plates ± standard deviation ("S.D.") from the mean. Qualitatively similar results were obtained in an additional experiment.

FIGURE 6A-P. The combination of Ad.*mda*-7 with AS K-*ras* PS ODN synergistically suppressed growth and decreased survival in mutated K-*ras* pancreatic carcinoma cells. Pancreatic carcinoma cell lines having a mutated K-*ras* gene (AsPC-1 [panels A,E,I,M], MIA PaCa-2 [panels B,F,J,N], and PANC-1 [panels C,G,K,O]) and pancreatic carcinoma cell line BxPC-3 (panels D,H,L,P), which has a wild-type *ras* gene, were treated as indicated and representative microscopic fields were photographed 3 days later. Cells were either untreated (control, panels A-D), treated with 0.5 μM AS K-*ras* PS ODN (panels E-H), infected with Ad.*mda*-7 (100 pfu/cell; panels I-L) or infected with Ad.*mda*-7 (100 pfu/cell) and then treated with 0.5 μM AS K-*ras* PS ODN (panels M-P).

FIGURE 7. AS K-*ras* PS ODN inhibited K-*RAS* protein synthesis in pancreatic carcinoma cells. The figure depicts Western blot analysis of K-*RAS* and EF-1α protein levels in cells treated with the various agents for three days. The concentration of MM, SC (scrambled) and AS PS ODN was 0.5 μM and the dose of virus was 100 pfu/cell.

FIGURE 8. The combination of Ad.*mda*-7 plus AS K-*ras* PS ODN or AS K-*ras* plasmids synergistically inhibited colony formation in mutated K-*ras* MIA PaCa-2 pancreatic carcinoma cells. The upper row of culture plates illustrates the effect of Ad.*mda*-7 plus AS K-*ras* PS ODN on MIA PaCa-2 colony formation. Cells were infected with 100 pfu/cell of Ad.*vec* (empty vector control) or Ad.*mda*-7, treated with 0.5 μM AS K-*ras* PS ODN plus 10 microliters lipofectamine, reseeded at a

density of 400 cells/plate and fixed and stained with Giemsa after three weeks. The lower row of culture plates illustrates the effect of Ad.*mda*-7 plus AS K-*ras* plasmid transfection on MIA PaCa-2 G418 resistant colony formation. Cells were infected with 100 pfu/cell of Ad.*vec* or Ad.*mda*-7, transfected with 10 micrograms of plasmid (either control pcDNA3.1 lacking insert, or the pcDNA3.1 vector containing a 346 nucleotide AS K-*ras* fragment), reseeded at a density of 2.5 x 10^5 cells/plate, selected in 400 μg/ml of G418 and then G418-resistant colonies were fixed and stained with Giemsa after three weeks.

FIGURE 9. Ad.*mda*-7 and AS K-*ras* PS ODN induced nucleosomal DNA degradation in K-*ras* mutant human pancreatic cancer cells. The indicated cell types were treated as indicated for 3 days. AS: 0.5 μM AS K-*ras* PS ODN; Ad.*mda*-7: 100 pfu/cell; Ad.*mda*-7 infected (100 pfu/cell) + 0.5 μM AS K-*ras* PS ODN. Nucleosomal ladder formation was determined as described in Kolch, 2000, Biochem. J. 351:289-305.

FIGURE 10A-D. *MDA*-7 protein was detected in mutated K-*ras* pancreatic carcinoma cells infected with Ad.*mda*-7 and treated with AS K-*ras* PS ODN. Cell lines PANC-1 (panel A), MIA PaCa-2 (panel B), AsPC-1 (panel C) and BxPC-3 (panel D) were treated for one day as indicated. In each of the panels, the rows contain cells treated as follows: 1 = control cells; 2 = AS K-*ras* PS ODN treated; 3 = Ad.*vec* treated; 4 = Ad.*vec* + AS K-*ras* PS ODN treated; 5 = Ad.*mda*-7 treated; 6 = Ad.*mda*-7 + MM PS ODN treated; 7 = Ad.*mda*-7 + SC PS ODN treated; 8 = Ad.*mda*-7 + AS K-*ras* PS ODN treated; 9 = PC-3 prostate carcinoma cells treated for one day with Ad.*vec*; 10 = PC-3 cells treated for one day with Ad.*mda*-7 (used as a positive control for *mda*-7 protein expression). Lysates of treated cells were evaluated by Western blotting for *MDA*-7 and *EF-1*α protein as described in Su et al., 1998, Proc. Natl. Acad. Sci. U.S.A. 95:14400-14405; Lebedeva et al., 2000, Cancer Res. 60:6052-6060; and Su et al., 1995, Intl. J. Oncol. 7:1279-1284. Arrowhead and bracket indicate *MDA*-7 proteins detected by Western blotting. The concentration of MM, SC and AS PS ODN was 0.5 μM and the dose of virus was 100 pfu/cell.

FIGURE 11. Expression of *mda*-7 mRNA in Ad.*mda*-7 infected mutated and wild-type K-*ras* pancreatic carcinoma cells. The indicated cell lines were treated for three days, total RNA was isolated and analyzed by Northern blotting for *mda*-7 and GAPDH mRNA. The concentration of SC, MM and AS PS ODN was 0.5 μM and the dose of virus was 100 pfu/cell.

FIGURE 12. Expression of *BAX, BCL-2* and *EF-1α* proteins in pancreatic carcinoma cells after various treatment protocols. The different cell lines were treated for three days as indicated and the levels of the respective proteins were determined using 30 μg of total protein lysates by Western blotting using the respective antibodies as described in Su et al., 1998, Proc. Natl. Acad. Sci. U.S.A. 95:14400-14405; Lebedeva et al., 2000, Cancer Res. 60:6052-6060; and Su et al., 1995, Intl. J. Oncol. 7:1279-1284. The concentration of MM, SC and AS PS ODN was 0.5 μM and the dose of virus was 100 pfu/cell.

FIGURE 13. Percent colony formation of MIA PaCa-2 cells which were, as represented by bars from left to right, either transfected with empty vector ("Vec"); transfected with vector containing a 346 K-*ras* fragment, in antisense orientation ("AS K-*ras* (346)"); transfected with vector containing a 631 K-*ras* fragment, in antisense orientation ("AS-K*ras* (631)"); transfected with empty vector and infected with Ad.*mda*-7 ("Vec + *mda*-7"); transfected with vector encoding AS K-*ras* (346) and infected with Ad.*mda*-7 ("AS K-*ras*(346) + *mda*-7"); or transfected with vector encoding AS K-*ras* (631) and infected with Ad.*mda*-7 ("AS K-*ras*(631) + *mda*-7").

FIGURE 14A-B. Controls for experiments described in Section 8. (A) Numbers of uninfected MIA PaCa-2 pancreatic carcinoma cells treated with either a lysate of hepatocytes infected with empty adenovirus (empty diamond; "Con + Ad.*vec* Lysate"); a lysate of hepatocytes infected with Ad.*mda*-7 (empty circle; "Con + Ad.*mda*-7 Lysate"); or untreated (empty square; "Con"). (B) Numbers of uninfected MIA PaCa-2 pancreatic carcinoma cells treated with either a culture supernatant of hepatocytes infected with empty adenovirus (empty diamond; "Con + Ad.*vec* Sup"); a culture supernatant of hepatocytes infected with Ad.*mda*-7 (empty circle; "Con + Ad.*mda*-7 Sup"); or untreated (empty square; "Con").

FIGURE 15A-B. (A) Numbers of MIA PaCa-2 pancreatic carcinoma cells, infected with Ad.*mda*-7, which were either untreated (empty square; "Ad.*mda*-7"), or treated with a lysate of hepatocytes infected with empty adenovirus (empty circle; "Ad.*mda*-7 + Ad.*vec* Lysate"); or a lysate of hepatocytes infected with Ad.*mda*-7 (square with + overstrike ; "Ad.*mda*-7 + Ad.*mda*-7 Lysate"); or by transfection with AS K-*ras* phosphorothioate-linked oligonucleotides ("PS ODN") (diamond with + overstrike; "Ad.*mda*-7 + AS K-*ras*"). (B) Numbers of MIA PaCa-2 pancreatic carcinoma cells, infected with Ad.*mda*-7, which were either untreated

(empty square; "Ad.*mda*-7"), or treated with a culture supernatant of hepatocytes infected with empty adenovirus (empty circle; "Ad.*mda*-7 + Ad.*vec* Sup"); or a culture supernatant of hepatocytes infected with Ad.*mda*-7 (empty triangle ; "Ad.*mda*-7 + Ad.*mda*-7 Sup"); or by transfection with AS K-*ras* PS ODN (empty diamond; "Ad.*mda*-7 + AS K-*ras*").

FIGURE 16A-B. (A) Numbers of MIA PaCa-2 pancreatic carcinoma cells, transfected with AS K-*ras* PS ODN, which were either untreated (empty square; "AS K-*ras*"), or treated with a lysate of hepatocytes infected with empty adenovirus (empty circle; "AS K-*ras* + Ad.*vec* Lysate"); or a lysate of hepatocytes infected with Ad.*mda*-7 (square with + overstrike ; "AS K-*ras* + Ad.*mda*-7 Lysate"); or by infection with Ad.*mda*-7 (diamond with + overstrike; "Ad.*mda*-7 + AS K-*ras*").

(B) Numbers of MIA PaCa-2 pancreatic carcinoma cells, transfected with AS K-*ras* PS ODN, which were either untreated (empty square; "AS K-*ras*"), or treated with a culture supernatant of hepatocytes infected with empty adenovirus (empty circle; "AS K-*ras*-7 + Ad.*vec* Sup"); a culture supernatant of hepatocytes infected with Ad.*mda*-7 (empty triangle ; "AS K-*ras* + Ad.*mda*-7 Sup"); or by infection with Ad.*mda*-7 (empty diamond; "AS K-*ras* + Ad.*mda*-7").

FIGURE 17A-D. Effects of Ad.*mda*-7 and Ad.K-*ras* AS, alone and in combination, on the growth of (A) AsPC-1; (B) BxPC-3; (C) PANC-1; and (D) MIA PaCa-2 pancreatic carcinoma cells.

FIGURE 18. Infection of pancreatic cancer cell lines with Ad.*bpv* results in the production of *mda*-7 mRNA.

FIGURE 19. Infection of Mia Paca-2 cells with Ad.*bpv* results in the production of MDA-7 protein.

FIGURE 20A-D. Infection of pancreatic cancer cell lines with Ad.*bpv* results in inhibition of growth of (A) AsPC-1, (B) BxPC-3, (C) PANC-1, and (D) MIA PaCa-2 pancreatic carcinoma cells. The data represent mean ± S.D. of quadruplicate samples from three independent experiments.

5. DETAILED DESCRIPTION OF THE INVENTION

For clarity of presentation, and not by way of limitation, the detailed description is divided into the following subsections:

(i) compositions that increase *MDA*-7 protein;

(ii) compositions that decrease *RAS* activity;

(iii) assays to identify suitable target cells;

(iv) assays to identify anti-cancer small molecules; and

(v) methods of use.

5.1. COMPOSITIONS THAT INCREASE *MDA*-7 PROTEIN

The first component necessary for practicing the methods of the invention is a means for increasing the amount of *MDA*-7 protein in a cancer cell and/or within a population of cancer cells. The convention of distinguishing between the gene and protein by using lower case versus capital letters is followed herein, so that *mda*-7 refers to nucleic acid molecules and *MDA*-7 refers to proteins (the same designations are used herein for *ras* genes and their encoded proteins). The term "gene" as used herein refers to any nucleic acid from which a functional protein can ultimately be derived, and encompasses, for example, genomic DNA as well as cDNA. The term "*MDA*-7" as used herein refers to a protein having essentially the amino acid sequence set forth as SEQ ID NO:2, having Genbank Accession Number U16261. A nucleic acid encoding *MDA*-7 may have the coding sequence as set forth in SEQ ID NO:1, Genbank Accession No. U16261, or another sequence which, when translated, produces a protein having essentially the same amino acid sequence. It should be noted that the portion of the nucleic acid sequence presented as SEQ ID NO:1 which constitutes the protein encoding region extends from nucleotide 275 to nucleotide 895. The scope of the invention embraces functional equivalents of the nucleic acid and protein which vary in insignificant ways from the native molecules; for example, it includes isolated nucleic acids which hybridize to the nucleic acid sequence set forth as SEQ ID NO:1 under stringent hybridization conditions, *e.g.*, hybridization in 0.5 M $NaHPO_4$, 7 percent sodium dodecyl sulfate ("SDS"), 1 mM ethylenediamine tetraacetic acid ("EDTA") at 65°C, and washing in 0.1x SSC/0.1 percent SDS at 68°C (Ausubel et al., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & Sons, Inc. New York, at p. 2.10.3), as well as the proteins encoded by such hybridizing sequences. It also includes nucleic acids having essentially the sequence set forth as SEQ ID NO:1, but modified to contain restriction sites appropriate for insertion into a particular expression vector.

The use of the term "increasing" does not presuppose that detectable levels of *MDA*-7 protein are constitutively present in the cell prior to treatment, such

that the level may be "increased" from an undetectable or 0 level. An increase in *MDA*-7 protein may be evaluated indirectly by detecting the presence of and/or quantifying the amount of *MDA*-7 encoding mRNA or directly by detecting the presence of and/or quantifying the amount of *MDA*-7 protein. *MDA*-7 encoding mRNA may be detected, for example, by Northern blot or by reverse transcription-polymerase chain reaction ("RT-PCR") (*e.g.* using *mda*-7 specific primers such as ATGCTCTGTCCCTGCAGATA (SEQ ID NO:3) and CTCTGGATGCTGTGAAGAGT (SEQ ID NO:4) as described in Jiang et al., 1995, Oncogene 11:2477-2486. *MDA*-7 protein may be detected by Western blot, for example using antibody directed against an *MDA*-7 specific peptide such as Pro-Ser-Gln-Glu-Asn-Glu-Met-Phe-Ser-Ile-Arg-Asp (SEQ ID NO:5; amino acid residues 153-164 of *MDA*-7 protein), also described in Jiang et al., 1995, Oncogene 11:2477-2486. In preferred embodiments, the amount of *MDA*-7 protein increases by a factor of at least 5, and more preferably by a factor of at least 10.

The amount of *MDA*-7 protein may be increased by increasing the amount of *mda*-7 encoding mRNA in a cancer cell. For instance, the amount of *MDA*-7 encoding mRNA may be increased by introducing, into the cell, an expression vector containing *MDA*-7 encoding nucleic acid, in an expressible form. An "expressible form" is one which contains the necessary elements for transcription and/or translation. For example, the *MDA*-7 encoding nucleic acid may be operatively linked to a suitable promoter element, and may comprise transcription initiation and termination sites, nucleic acid encoding a nuclear localization sequence, ribosome binding sites, polyadenylation sites, mRNA stabilizing sequences, etc..

For example, where *mda*-7 nucleic acid is to be transcribed into RNA, the nucleic acid may be operatively linked to a suitable promoter element, for example, but not limited to, the cytomegalovirus immediate early promoter, the Rous sarcoma virus long terminal repeat promoter, the human elongation factor 1α promoter, the human ubiquitin c promoter, etc.. It may be desirable, in certain embodiments of the invention, to use an inducible promoter. Non-limiting examples of inducible promoters include the murine mammary tumor virus promoter (inducible with dexamethasone); commercially available tetracycline-responsive or ecdysone-inducible promoters, etc.. In specific non-limiting embodiments of the invention, the promoter may be selectively active in cancer cells; one example of such a promoter is the PEG-3 promoter, as described in International Patent Application No.

PCT/US99/07199, Publication No. WO 99/49898 (published in English on October 7, 1999); other non-limiting examples include the prostate specific antigen gene promoter (O'Keefe et al., 2000, Prostate 45:149-157), the kallikrein 2 gene promoter (Xie et al., 2001, Human Gene Ther. 12:549-561), the human alpha-fetoprotein gene promoter (Ido et al., 1995, Cancer Res. 55:3105-3109), the c-*erbB*-2 gene promoter (Takakuwa et al., 1997, Jpn. J. Cancer Res. 88:166-175), the human carcinoembryonic antigen gene promoter (Lan et al., 1996, Gastroenterol. 111:1241-1251), the gastrin-releasing peptide gene promoter (Inase et al., 2000, Int. J. Cancer 85:716-719). the human telomerase reverse transcriptase gene promoter (Pan and Koenman, 1999, Med. Hypotheses 53:130-135), the hexokinase II gene promoter (Katabi et al., 1999, Human Gene Ther. 10:155-164), the L-plastin gene promoter (Peng et al., 2001, Cancer Res. 61:4405-4413), the neuron-specific enolase gene promoter (Tanaka et al., 2001, Anticancer Res. 21:291-294), the midkine gene promoter (Adachi et al., 2000, Cancer Res. 60:4305-4310), the human mucin gene *MUC1* promoter (Stackhouse et al., 1999, Cancer Gene Ther. 6:209-219), and the human mucin gene *MUC4* promoter (Genbank Accession No. AF241535), which is particularly active in pancreatic cancer cells (Perrais et al., 2001, published on June 19, 2001 by J Biol. Chem., "JBC Papers in Press" as Manuscript M104204200).

Suitable expression vectors include virus-based vectors and non-virus based DNA or RNA delivery systems. Examples of appropriate virus-based gene transfer vectors include, but are not limited to, those derived from retroviruses, for example Moloney murine leukemia-virus based vectors such as LX, LNSX, LNCX or LXSN (Miller and Rosman, 1989, Biotechniques 7:980-989); lentiviruses, for example human immunodeficiency virus ("HIV"), feline leukemia virus ("FIV") or equine infectious anemia virus ("EIAV")-based vectors (Case et al., 1999, Proc. Natl. Acad. Sci. U.S.A. 96: 22988-2993; Curran et al., 2000, Molecular Ther. 1:31-38; Olsen, 1998, Gene Ther. 5:1481-1487; United States Patent Nos. 6,255,071 and 6,025,192); adenoviruses (Zhang, 1999, Cancer Gene Ther. 6(2):113-138; Connelly, 1999, Curr. Opin. Mol. Ther. 1(5):565-572; Stratford-Perricaudet, 1990, Human Gene Ther. 1:241-256; Rosenfeld, 1991, Science 252:431-434; Wang et al., 1991, Adv. Exp. Med. Biol. 309:61-66; Jaffe et al., 1992, Nat. Gen. 1:372-378; Quantin et al., 1992, Proc. Natl. Acad. Sci. U.S.A. 89:2581-2584; Rosenfeld et al., 1992, Cell 68:143-155; Mastrangeli et al., 1993, J. Clin. Invest. 91:225-234; Ragot et al., 1993, Nature 361:647-650; Hayaski et al., 1994, J. Biol. Chem. 269:23872-23875; Bett et

al., 1994, Proc. Natl. Acad. Sci. U.S.A. 91:8802-8806), for example Ad5/CMV-based E1-deleted vectors (Li et al., 1993, Human Gene Ther. 4:403-409); adeno-associated viruses, for example pSub201-based AAV2-derived vectors (Walsh et al., 1992, Proc. Natl. Acad. Sci. U.S.A. 89:7257-7261); herpes simplex viruses, for example vectors based on HSV-1 (Geller and Freese, 1990, Proc. Natl. Acad. Sci. U.S.A. 87:1149-1153); baculoviruses, for example AcMNPV-based vectors (Boyce and Bucher, 1996, Proc. Natl. Acad. Sci. U.S.A. 93:2348-2352); SV40, for example SVluc (Strayer and Milano, 1996,Gene Ther. 3:581-587); Epstein-Barr viruses, for example EBV-based replicon vectors (Hambor et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:4010-4014); alphaviruses, for example Semliki Forest virus- or Sindbis virus-based vectors (Polo et al., 1999, Proc. Natl. Acad. Sci. U.S.A. 96:4598-4603); vaccinia viruses, for example modified vaccinia virus (MVA)-based vectors (Sutter and Moss, 1992, Proc. Natl. Acad. Sci. U.S.A. 89:10847-10851) or any other class of viruses that can efficiently transduce human tumor cells and that can accommodate the nucleic acid sequences required for therapeutic efficacy.

Non-limiting examples of non-virus-based delivery systems which may be used according to the invention include, but are not limited to, so-called naked nucleic acids (Wolff et al., 1990, Science 247:1465-1468), nucleic acids encapsulated in liposomes (Nicolau et al., 1987, Methods in Enzymology 1987:157-176), nucleic acid/lipid complexes (Legendre and Szoka, 1992, Pharmaceutical Research 9:1235-1242), and nucleic acid/protein complexes (Wu and Wu, 1991, Biother. 3:87-95).

MDA-7 may also be produced by yeast or bacterial expression systems. For example, bacterial expression may be achieved using plasmids such as pCEP4 (Invitrogen, San Diego, CA), pMAMneo (Clontech, Palo Alto, CA; see *below*), pcDNA3.1 (Invitrogen, San Diego, CA), etc..

Depending on the expression system used, nucleic acid may be introduced by any standard technique, including transfection, transduction, electroporation, bioballistics, microinjection, etc..

In preferred, non-limiting embodiments of the invention, the expression vector is an E1-deleted human adenovirus vector of serotype 5. To prepare such a vector, an expression cassette comprising a transcriptional promoter element operatively linked to an *MDA*-7 coding region and a polyadenylation signal sequence may be inserted into the multiple cloning region of an adenovirus vector shuttle plasmid, for example pXCJL.1 (Berkner, 1988, Biotechniques 6:616-624). In the

context of this plasmid, the expression cassette may be inserted into the DNA sequence homologous to the 5' end of the genome of the human serotype 5 adenovirus, disrupting the adenovirus E1 gene region. Transfection of this shuttle plasmid into the E1-transcomplementing 293 cell line (Graham et al., 1977, J. General Virology 36:59-74), or another suitable cell line known in the art, in combination with either an adenovirus vector helper plasmid such as pJM17 (Berkner, 1988, Biotechniques 6:616-624; McGrory et al., 1988, Virology 163:614-617) or pBHG10 (Bett et al., 1994, Proc. Natl. Acad. Sci. U.S.A. 91: 8802-8806) or a ClaI-digested fragment isolated from the adenovirus 5 genome (Berkner, 1988, Biotechniques 6:616-624), allows recombination to occur between homologous adenovirus sequences contained in the adenovirus shuttle plasmid and either the helper plasmid or the adenovirus genomic fragment. This recombination event gives rise to a recombinant adenovirus genome in which the cassette for the expression of the foreign gene has been inserted in place of a functional E1 gene. When transcomplemented by the protein products of the human adenovirus type 5 E1 gene (for example, as expressed in 293 cells), these recombinant adenovirus vector genomes can replicate and be packaged into fully-infectious adenovirus particles. The recombinant vector can then be isolated from contaminating virus particles by one or more rounds of plaque purification (Berkner, 1988, Biotechniques 6:616-624), and the vector can be further purified and concentrated by density ultracentrifugation.

In a specific, non-limiting embodiment of the invention, an *mda-7* nucleic acid, in expressible form, may be inserted into the modified Ad expression vector pAd.CMV (Falck-Pedersen et al., 1994, Mol. Pharmacol. 45:684-689).

This vector contains, in order, the first 355 base pairs from the left end of the adenovirus genome, the cytomegalovirus immediate early promoter, DNA encoding splice donor and acceptor sites, a cloning site for the *mda-7* gene, DNA encoding a polyadenylation signal sequence from the β globin gene, and approximately three kilobase pairs of adenovirus sequence extending from within the E1B coding region. This construct may then be introduced into 293 cells (Graham et al., 1977, J. Gen. Virol. 36:59-72) together with plasmid JM17 (above), such that, as explained above, homologous recombination can generate a replication defective adenovirus containing *MDA-7* encoding nucleic acid. FIGURE 2A shows the *mda-7* gene, inserted into the pAd.CMV vector, forming pCMV/*mda-7*; FIGURE 2B shows

the recombination event (curved lines) between the JM17 plasmid and pCMV/*mda*-7; and FIGURE 2C shows the resulting Ad.*mda*-7 replication defective adenovirus.

In another specific, non-limiting embodiment of the invention, where *mda*-7 is to be introduced into cells in culture, a suitable expression vector may be prepared by inserting an *mda*-7 nucleic acid, extending from nucleotide 176 to nucleotide 960 in the sequence presented as SEQ ID NO:1, encoding the open reading frame, into pCEP4 (Invitrogen, San Diego, CA) downstream of the CMV promoter. Another suitable vector may be the Rous sarcoma virus ("RSV") vector available as pREP4 (Invitrogen).

In alternative embodiments, the amount of *MDA*-7 encoding mRNA may be increased by increasing expression of the *mda*-7 gene endogenous to the cancer cells. For example, such increased expression may be induced by exposing the cells to one or more differentiation-promoting agent. As one non-limiting specific example, the cancer cells may be exposed to effective concentrations of IFN-β and mezerein (see, for example, Jiang and Fisher, 1993, Mol. Cell. Different. 1(3):285-299, which exposed cells, in culture, to 2000 units/ml of IFN-β and 10 ng/ml of mezerein). Alternatively, the cancer cells may be exposed to an effective amount of a small molecule identified as set forth in Section 5.4.

In further embodiments, the amount of *MDA*-7 in a cancer cell and/or within a population of cancer cells may be increased by introducing *MDA*-7 protein into the cell and/or population. For example, for introduction into a cell, *MDA*-7 protein could be incorporated into a microparticle for uptake by pinocytosis or phagocytosis. *MDA*-7 protein may be introduced into a population such that it is present in the extracellular environment of the cells; there is evidence that *MDA*-7 is a secreted protein and, as such, may be biologically active in the extracellular context; support for biological activity of extracellularly administered *MDA*-7 is presented in Section 8, below. For embodiments in which *MDA*-7 protein is introduced into a cell or population of cells, *MDA*-7 may be comprised, for example, in microparticles, liposomes, or other protein-stabilizing formulations known in the art.

5.2. COMPOSITIONS THAT DECREASE *RAS* ACTIVITY

The second component necessary for practicing the methods of the invention is a means for decreasing *RAS* activity in a cancer cell and/or within a population of cancer cells. This decrease in activity may be achieved through either

genetic means (e.g. the application of antisense, triplex or ribozyme technologies to decrease the transcription or translation of the *ras* gene or its message, or to decrease the transcription or translation of another molecule or molecules within the *RAS* pathway), or through pharmacological means (e.g. the use of small molecular inhibitors of the *RAS* pathway or the use of farnesyl transferase inhibitors to impair the association of *RAS* with the cell membrane).

The term "*RAS*" as used herein refers to members of the *RAS* family of proteins, including the proteins human H-*RAS*, K-*RAS*, and N-*RAS* and the corresponding genes H-*ras*, K-*ras* and N-*ras*, having sequences as set forth in Genbank Accession No. J00277, Genbank Accession No. M54968 and Genbank Accession No. XM 001317, respectively, incorporated by reference herein, as well as mutant forms. The nucleic acid sequence of wild-type K-*ras* and its encoded amino acid sequence are set forth as SEQ ID NOS: 6 and 7, respectively. The mutant forms of *RAS* include those having point mutations at amino acid residues 12, 13, 18 and/or 61. Accordingly, the *RAS* proteins encompassed by the present invention comprise the aforementioned amino acid sequences and those sequences having any amino acid substituted at position 12, 13, 18, and/or 61. The scope of the invention also includes nucleic acids encoding said amino acid sequences. SEQ ID NOS: 8-11 are amino acid sequences of K-*RAS* having single amino acid substitutions at each of these positions, where Xaa can represent any amino acid. In specific non-limiting embodiments, the amino acid at position 12 of K-*ras* may be substituted with aspartic acid (Xaa = Asp), glycine (Xaa = Gly), valine (Xaa = Val), or arginine (Xaa = Arg).

The term "*ras*" also applies, with regard to nucleic acids (including RNA and DNA molecules), to nucleic acid molecules which hybridize to a nucleic acid sequence as set forth in Genbank Accession number J00277, SEQ ID NO:6, or Genebank Accession No. XM 001317, under stringent hybridization conditions *e.g.*, hybridization in 0.5 M $NaHPO_4$, 7 percent sodium dodecyl sulfate ("SDS"), 1 mM ethylenediamine tetraacetic acid ("EDTA") at 65°C, and washing in 0.1x SSC/0.1 percent SDS at 68°C (Ausubel et al., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & Sons, Inc. New York, at p. 2.10.3) and to proteins which they encode. The present invention provides for the coding strand nucleic acid molecule, as well as its complementary (antisense strand), and oligonucleotide portions thereof. Oligonucleotides may preferably be 5-50 bases or base-pairs in length.

The aforedescribed *ras* nucleic acid molecules (including oligonucleotides) may be comprised in larger nucleic acid molecules, for example appropriate vector molecules, wherein they may be in "expressible form" as defined above. Further, the nucleic acid molecules encompassed herein may be altered to comprise non-naturally occurring nucleic acids or have stabilized (nuclease resistant) linkages.

Examples of modified base moieties which may be used include, but are not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-carboxyhydroxymethyluracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, ßgalactosylqueosine, inosine, N6-isopentyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methyl cytosine, 5-methyl cytosine, N6-adenine,7 methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, ß-D-mannosylqueosine, 5'-methoxycarboxymethylyuracil, 5-methoxyuracil, 2-methylthio-N6-isopentyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thioruracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid(v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl)uracil, (acp3)w, and 2,6-diaminopurine.

One preferred non-limiting example of a modified linkage is a phosphorothioate internucleoside linkage, *e.g.* as described in United States Patent No. 6,242,589 by Cook and Manoharab, issued June 5, 2001. Other nucleoside linkages resistant to nuclease digestion include phosphotriester, methyl phosphonate, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages (see United States Patent No. 6,229,006 by Wu, issued May 8, 2001). Peptide nucleic acids may also be used, as described, for example, in United States Patent No. 5,539,082 by Nielsen et al., issued July 23, 1996; United States Patent No. 5,714,331 by Buchardt et al., issued February 3, 1998; United States Patent No. 5,719,262 by Buchardt et al., issued February 11, 1998, and in Nielsen et al., 1991, Science 254:1497-1500.

The phrase "a decrease in *RAS* activity", as used herein, indicates a decrease in the amount or proportion of one or more species of molecule within the *RAS* pathway which is (are) in an activated state. A simplified diagram of the *RAS*

pathway, showing certain (but not all) molecules upstream and downstream of *RAS* which may reflect *RAS* activity according to the invention, is presented in FIGURE 1. For certain members of the *RAS* pathway, an active state is reflected by phosphorylation. The "decrease" is relative to the amount or proportion of activated molecules in a cell or population of cells which has (have) not been treated according to the invention. As one specific, non-limiting example, *RAS* protein is in an active state when it is bound to GTP. Where the invention produces a decrease in proportion of *RAS* molecules in the active state, there has been a decrease in *RAS* activity.

Other molecules in the *RAS* pathway which can reflect and/or can be inhibited to produce a "decrease in *RAS* activity" as defined herein include, but are not limited to, the EGF receptor, *RAF*1 (*"RAF"*), PI3 kinase , MAPK kinase, MAP kinase (*"MAPK"*), *MEKK1*, and the MAPK proteins *ERK1* and *ERK2*. As one specific non-limiting example, a decrease in *RAS* activity may be reflected by a decrease in phosphorylation of MAPK kinase, wherein the active form of that molecule is phosphorylated, and *RAS* activity as defined herein may be decreased by inhibiting MAPK kinase. The "decrease in *RAS* activity" may also be reflected by the decrease in activation of an as yet unidentified or unconfirmed *RAS* effector molecule. This document incorporates the definition of a *RAS* effector molecule articulated in Crespo and León, 2000, Cell. Mol. Life Sci. 57:1613-1636, which states that a *RAS* effector molecule exhibits "(1) preferential binding to the GTP-bound form of *RAS*, (2) binding to a region within the effector domain, this binding eliciting the activation of the effector molecule with a subsequent biochemical and/or biological effect, and (3) dysfunction of the putative effector molecule abolishes at least part of the *RAS*-mediated effects".

In preferred embodiments, *RAS* activity may be decreased by inhibiting *RAS* protein synthesis using antisense technology. "Antisense *ras*" molecules may be used to interfere with *ras* RNA function at various stages, including splicing, catalytic activity, translocation of RNA to the site of protein translation, and/or translation of protein from the RNA (see, for example, United States Patent No. 6,255,111 by Bennett et al., issued July 3, 2001, which provides a review of antisense techniques and materials). According to the present invention, the translation of *ras*-specific messenger RNA (mRNA) may be blocked through the introduction into cells of synthetic nucleic acid sequences that are complementary to all or part of the endogenous gene. These synthetic nucleotide sequences interact with endogenous

mRNA sequences based on their sequence complementarity, forming double-stranded RNA species that are less able to be translated into protein species and more prone to degradation by the enzyme RNaseH than single-stranded RNA. This approach, or variants thereof, have been shown to be successful in inhibiting proliferation of a number of human cancer cell lines *in vitro* (Aoki et al., 1997, Molecular Carcinogenesis 20:251-258; Kita et al., 1999, Intl. Journal of Cancer 80:553-558) and suppressing the growth of human tumors *in vivo* in animal models (Nakano et al., 2001, Molecular Ther. 3:491-499).

An antisense molecule may be designed to target *ras* mRNA, for example, in the region encompassing the translation initiation or termination codon of the open reading frame. In preferred embodiments, the antisense molecule is an oligonucleotide between about 6 and 50 bases in length, and complementary to a portion of *ras* mRNA such that hybridization with the antisense oligonucleotide inhibits or prevents translation to form *RAS* protein. In specific, non-limiting embodiments an antisense oligonucleotide hybridizes to a wild-type or mutated *ras* nucleic acid under stringent conditions, as defined above. Although antisense oligonucleotides complementary to the 5' end of *ras* mRNA, for example the 5' untranslated sequence up to and including the AUG initiation codon, are preferred, oligonucleotides complementary to the 3' untranslated sequences or, less preferably, the coding regions of *ras* mRNA, may also be used. Where *ras* contains a point mutation, mutation-specific oligonucleotides may be employed, as such oligonucleotides may selectively inhibit expression of the mutated, but not the wild type, *ras* gene.

Specific, non-limiting examples of viral vectors that encode antisense *ras* nucleic acids are known in the art (*e.g.*, AS-K-*ras*-LNSX (Aoki et al., 1995, Cancer Res. 55:3810-3816); AχCA-AS-K-*ras* (Nakano et al., 2001, Mol. Ther. 3(4):491499)). In specific, non-limiting embodiments of the invention, a replication defective adenovirus vector encoding antisense *ras* may be used. Such vectors may be prepared using methods analogous to those used to prepare Ad.*mda*-7. FIGURE 3A-C show the preparation of one specific example of such a vector, in which a 631 nucleotide sequence representing nucleotides 172 to 802 of K-*ras*, in antisense orientation, is inserted in pAd.CMV to form pCMV/K-*ras* AS (FIGURE 3A). Homologous recombination with pJM17 in 293 cells (FIGURE 3B) may be used to generate replication defective Ad.K-*ras* AS virus (FIGURE 3C). The effectiveness of

an adenovirus vector encoding antisense *ras* sequences, used together with Ad.*mda*-7, is demonstrated by data presented in Section 9, below. In another specific embodiment, a pcDNA3.1 (neomycin resistance) expression vector containing a 346 nucleotide K-*ras* gene fragment (from nt 172 to 517), as described below in Section 6, may be used.

Specific, non-limiting examples of antisense *ras* oligonucleotides are known in the art, for example the following phosphorothioate antisense oligonucleotides targeting mutations at codon 12 of K-*ras* (mutant specific sequence underlined):

for mutation to GTT, 5'-CTACGCCAACAGCTCCA-3' (SEQ ID NO:12);

for mutation to CGT, 5'-CTACGCCACGAGCTCCA-3' (SEQ ID NO:13); and

for mutation to GAT, 5'-CTACGCCATCAGCTCCA-3' (SEQ ID NO:14)

(Kita et al., 1999, Int. J. Cancer 80:553-558).

In a preferred specific embodiment, the phophorothioate oligonucleotide 5'-CACAAGTTTATATTCAGT -3' (SEQ ID NO:15), complementary to K-*ras* nucleotides 196 - 213 (adjacent to the start codon), as described below in Section 6, or an oligonucleotide comprising SEQ ID NO:15 or hybridizable to the complement of SEQ ID NO:15 under stringent conditions, may be used.

A vector containing sequence expressible to form antisense *ras* nucleic acid may be introduced into a cancer cell/cancer cell population by methods known in the art, such as infection, transfection, electroporation, etc..

Antisense *ras* oligonucleotides may be introduced into a cancer cell/cancer cell population by methods known in the art, which may utilize, for example, liposomes (*e.g.* DC-cholesterol liposomes, cationic liposomes, liposomes containing Sendai virus coat protein), imidazolium lipids (see, for example, United States Patent No. 6,245,520 by Wang et al., issued June 12, 2001), cationic lipids (see, for example, United States Patent No. 6,235,310 by Wang et al., issued May 22, 2001), lipofection, asialoglycoprotein poly(L)lysine complexes, and microbubbles (see, for example, United States Patent No. 6,245,747 by Porter et al., issued June 12,

2001). See, for example, the experiments described in Section 6, below, where lipofectamine was used to promote oligonucleotide uptake.

In particular, non-limiting embodiments of the invention, a single vector may be used for the introduction of both an *MDA*-7-encoding nucleic acid and antisense *ras* sequences. For example, but not by way of limitation, an expressible form of *mda*-7 nucleic acid and *ras* antisense encoding nucleic acid sequences, operatively linked to the same promoter, to two promoters which are the same, or to two promoters which are different, may be incorporated into an E1-deleted human adenovirus vector of serotype 5, via, for example, pAd.CMV, as described above. FIGURE 4 presents a diagram showing the preparation of such a vector. Briefly, HEK-293 cells may be transfected with overlapping DNA fragments from the viral genome of Ad.*mda*-7 and from plasmid pPF446 (Volker and Young, 1983, Virol. 125:175-193) modified to contain the K-*ras* sequence, cloned in an antisense orientation, in the E3 region. Recombination (shown by a curved line) between the two fragments generates a genome containing both *mda*-7 and AS K-*ras* sequences.

In other non-limiting embodiments, ribozymes may be used to decrease *RAS* activity. Ribozymes are enzymatic RNA molecules which catalyze the specific cleavage of RNA. Ribozyme molecules according to the invention comprise a region complementary to *ras* mRNA where the region is capable of specifically hybridizing to *ras* mRNA, and may have a hairpin or hammerhead structure (Rossi, 1994, Current Biology 4:469-471; United States Patent No. 5,093,246 by Cech et al., issued March 3, 1992; Haseloff and Gerlach, 1988, Nature 334:585-591; Zaug et al., 1984, Science 224:574-578; Zaug and Cech, 1986, Science 231:470-475; Zaug et al., 1986, 324:429-433; Been and Cech, 1986, Cell 47:207-216).

In further embodiments, *ras* gene transcription may be blocked by targeted deoxyribonucleotide sequences complementary to the *ras* gene regulatory region which produce triple helical structures that prevent transcription (Helene, 1991, Anticancer Drug. Des. 6(6):569-584; Helene et al., 1992, Ann. N.Y. Acad. Sci. 660:27-36; Maher, 1992, Bioassays 14(12):807-815).

In further embodiments, *RAS* activity may be decreased by introducing, into a cancer cell, a dominant negative *ras* mutant (*e.g.*, the H-*ras* mutant N116Y; Shichinohe, 1996, J. Surg. Res. 66:125-130).

In further embodiments of the invention, *RAS* activity may be decreased by administering an effective amount of a small molecule which may be identified using the methods set forth in Section 5.4, below.

In additional embodiments of the invention, *RAS* activity may be decreased by a farnesyl transferase inhibitor. In order to become activated through GTP binding, the cytosolic form of *RAS* must be localized to the inner surface of the cell membrane. This process is regulated by at least three types of post-translational modification which are performed on the *RAS* protein. One of these is the process of farnesylation, whereby a fatty acid farnesyl moiety is enzymatically attached to the cysteine residue in the CAAX motif located near the C-terminus of the protein. Inhibition of this reaction, which is catalyzed by the enzyme farnesyl transferase (FT), can drastically reduce *RAS* activation and block *RAS*-mediated transformation (Kohl et al., 1993, Science 260:1934 -1937; Kohl et al., 1994, Proc. Natl. Acad. Sci. U.S.A.91:9141-9145) . FT inhibitors fall into several classes. The first are those competing with the farnesyl moiety. These compounds include limonene, perillyl alcohol and perillic acid among others (reviewed in Hardcastle et al., 1999, Biochem. Pharm. 57:801-809). A second class includes agents that mimic the CAAX motif present in the *RAS* protein. These agents are referred to as peptidomimetics and include FT1-276 (Lantry et al., 2000, Carcinogenesis 2:113-116) , FTI-277 (Adjei et al., 2000, Clin Can Res 6:2318-2325), and L-739,749 (Lebowitz et al., 1995, Mol Cell Biol 15:6613-6622) among other compounds. The third class is comprised of compounds that combine the characteristics of both aforementioned groups, and are referred to as bisubstrate inhibitors. Compounds in this class include BMS-186511 (Schlitzer and Sattler, 2000, Eur J Med Chem 2000;35:721-726). *RAS* activity may be decreased by administering an effective amount of any of the foregoing compounds or other farnesyl transferase inhibitors known in the art. Such compounds may be administered orally, intravenously or by intratumoral injection among other routes.

5.3. ASSAYS TO IDENTIFY SUITABLE TARGET CELLS

A "target cell" is defined herein as a cancer cell which, when subjected to the methods of the invention, is expected to exhibit inhibited proliferative ability and/or characteristics of programmed cell death ("apoptosis").

In specific, preferred non-limiting embodiments of the invention, the present invention is directed toward inhibiting the proliferation and survival of

pancreatic cancer cells. Experimental data presented in Section 6, below, indicate that the synergistic effect of *MDA-7* and *ras* antisense molecules is observed in pancreatic cancer cells having a mutation activated *ras* gene, but not in cells having wild type *ras*. It therefore is desirable, although not required, to ascertain whether *RAS* activity is increased in a pancreatic cancer cell before applying the methods of the invention.

As the methods of the invention may be directed toward cancer cells which have origins other than the pancreas, this section provides guidelines for identifying suitable target cells. Suitable non-pancreatic cancer target cells exhibit increased *RAS* activity, and may be identified, for example, as having a mutation in a *ras* gene. For example, but not by way of limitation, cancers which have been associated with a *ras* gene mutation which increases *RAS* activity include malignant melanoma (Demunter et al., 2001, Cancer Res. 61:4916-4922), gastric cancer (Hao et al., 1998, J. Tongji Med. Univ. 18(3):141-144), gallbladder carcinoma (Kim et al., 2001, Cancer Lett. 169(1):59-68), colon carcinoma (Clarke et al., 2001, Int. J. Colorectal Dis. 16(2):108-111), lung cancer, particularly non-small cell lung cancer (Noda et al., 2001, Oncol. Rep. 8(4):889-92), acute lymphoblastic leukemia (Nakao et al., 2000, Leukemia 14(2):312-315), hepatocellular carcinoma (Weihrauch et al., 2001, Br. J. Cancer 84(7):982-989), liver angiosarcoma (Marion et al., 1991, Mol. Carcinog. 4(6):450-454); multiple myeloma (Tanaka et al., 1992, Int. J. Hematol. 56(2):119-127), bladder carcinoma (Saito et al., 1997, Int. J. Urol. 4(2):178-185), peripheral nerve sheath tumors (Watanabe et al., 2000, Int. J. Mol. Med. 5(6):605-608), childhood brain tumors (Maltzman et al., 1997, Cancer Epidemio. Biomarkers Prev. 6(4):239-243), ovarian tumors (Varras et al., 1999, Oncology 56(2):89-96), cervical carcinoma (Grendys et al., 1997, Gynecol. Oncol. 65(2):343-347), thyroid carcinoma (Basolo et al., 2000, Thyroid 10(1):19-23), chondrosarcoma (Sakamoto et al., 2001, Mod. Pathol. 14(4):343-349), and primary sarcoma of the heart (Garcia et al., 2000, Br. J. Cancer 82(6):1183-1185). Miyakis et al. (1998, Biochem. Biophys. Res. Commun. 251(2):609-612) reports a low incidence of *ras* point mutation in breast cancer subjects, but observed overexpression of *ras* in 67 percent of breast cancer specimens studied.

The presence of a *ras* mutation in a cancer cell, for example a cancer cell collected from a human subject, may be determined using standard laboratory techniques, such as, for example, PCR-Restriction Fragment Length Polymorphism ("PCR-RFLP") analysis, PCR-Single Strand Conformational Polymorphism ("PCR-

SSCP") analysis, LightCycler technology (analysis of melting temperatures of PCR products; see Nakao et al., 2000, Leukemia 14(2):312-315); fluorescent oligonucleotide ligation (Eggerding, 2000, Mol. Biotechnol. 14(3):223-233); PCR/dot blot hybridization (Grendys et al., 1997, Gynecol. Oncol. 65(2):343-347), PCR/restriction enzyme treatment/dot blot hybridization (Saito et al., 1997, Int. J. Urol. 4(2):178-185), PCR-primer introduced restriction with enrichment of mutant alleles ("PCR-PIREMA" assay; Basolo et al., 2000, Thyroid 10(1):19-23) and/or DNA sequencing.

Overexpression of *ras* may be determined using standard techniques, including Northern blot analysis and RT-PCR (Miyakis et al., 1998, Biochem. Biophys. Res. Commun. 251(2):609-612).

Where a representative cell line for a cancer cell is available, it may further be desirable to increase *MDA-7* and decrease *RAS* activity in a culture of said cell line, for example using techniques as described for the pancreatic carcinoma cell lines set forth in Section 6, *below*. Suitability of the cancer cell as a target cell would be indicated by a decrease in cell proliferation, a decrease in colony formation in soft agar, an increase in cell death (*e.g.*, apoptotic cell death), and/or a decrease in tumorigenicity (*e.g.*, in nude mice). For example, apoptotic cell death may be measured by methods known in the art, for example the TUNEL method, as described in Gravieli et al., 1992, J. Cell Biol. 119:493-501. In specific non-limiting embodiments of the invention, an increase in apoptosis may be indicated by an increase in the level of *BAX* protein and/or a decrease in *BCL-2* protein; for example, preferably, *BAX* may increase at least about 3-fold, and/or *BCL-2* may be reduced at least 1.2-fold, and/or the *BAX/BCL-2* ratio may increase by at least about 2-fold (Madireddi et al., 2000, Adv. Exptl. Med. Biol. 465:239-261; Su et al., 1998, Proc. Natl. Acad. Sci. U.S.A. 95:14400-14405; Saeki et al., 2000, Gene Ther. 7:2051-2057).

In particular embodiments, the present invention provides for a method for identifying a suitable cancer cell for treatment with *mda-7*/anti-*RAS* combination therapy, comprising (i) administering, to a test cancer cell, a first agent which increases the amount of *MDA-7* protein in combination with a second agent that decreases *RAS* activity in the cancer cell; (ii) determining whether the cancer cell exhibits at least one characteristic of apoptosis; wherein the presence of a characteristic of apoptosis has a positive correlation with the suitability of the cancer cell for treatment with *mda-7*/anti-*RAS* combination therapy.

In other particular embodiments, the present invention provides for a method for identifying a suitable cancer cell for treatment with *mda*-7/anti-*RAS* combination therapy, comprising (i) administering, to a culture of test cancer cells, a first agent which increases the amount of *MDA*-7 protein in combination with a second agent that decreases *RAS* activity; (ii) measuring the proliferation of cancer cells in the culture; and (iii) comparing the proliferation of cells measured in step (ii) with the proliferation of control cultures of the cancer cells in the presence of the same concentration of first agent or second agent, used alone; wherein if the combination of first and second agent results in a decrease in cell proliferation which is greater than the additive effect of the first agent and the second agent used alone, the cancer cell is suitable for treatment with *mda*-7/anti-*RAS* combination therapy.

5.4. ASSAYS TO IDENTIFY ANTI-CANCER SMALL MOLECULES

The present invention further provides for methods of identifying small molecules that may be effective in the treatment of cancers exhibiting an increase in *RAS* activity.

In particular non-limiting embodiments, compounds that decrease *RAS* activity could be identified by the following method. A *mda*-7 gene, in expressible form (*e.g.*, Ad.*mda*-7), could be introduced into a cancer cell line having increased *RAS* activity (*e.g.*, a pancreatic cancer cell line having an activating mutation in K-*ras*) to form a population of "test cells". The level of *MDA*-7 RNA or protein (or the absence of detectable *MDA*-7) in the test cells may be determined, and then the test cells may be exposed to various chemical or biochemical compounds, for example a combinatorial library of small molecules (also referred to herein as small molecules having a defined structure), by the addition of one or more compound to the culture media of parallel cultures of test cells. The appearance of *MDA*-7-encoding RNA or protein, or an increase in *MDA*-7-encoding RNA or protein, within the test cells and/or in the culture media would suggest that a chemical being tested may have decreased *RAS* activity in the test cells. Without being bound by any particular theory, this conclusion would be supported by the observation that activation of K-*ras* appears to interfere with the ability of the *mda*-7 RNA to be translated into MDA-7 protein (see Section 6, below). A chemical positively identified through this assay procedure may then be further tested for its ability to decrease *RAS* activity and/or to inhibit the proliferation and/or survival of cancer cells. Further, such a chemical may

be characterized to better define the mechanism whereby they exert their effects on activated K-*ras*.

Alternatively, pancreatic cancer cells or immortalized cell lines exhibiting an increase in *RAS* activity (*e.g.*, as a result of a *ras* mutation) may be treated with compounds known to decrease *RAS* activity (*e.g.*, antisense *ras* molecules, to produce a population of test cells. The level of *MDA*-7 (if any is detectable)-encoding RNA or protein in the test cells may be determined. The test cells then may be exposed to various chemical or biochemical compounds, for example a combinatorial library of small molecules, by the addition of one or more compound to the culture media of parallel cultures of test cells. The appearance of *MDA*-7-encoding RNA or protein, or an increase in *MDA*-7-encoding RNA or protein, within the test cells and/or in the culture media would suggest that a chemical being tested may have enhanced the activity of *MDA*-7 in the test cells. Alternatively or in addition, biological endpoints such as growth suppression or apoptosis may be used in the screening procedure. A chemical positively identified through this assay procedure may then be further characterized to determine its anti-cancer potential and/or to better define the mechanism whereby it exerts its effect on these cellular processes.

5.5. METHODS OF USE

The present invention relates to methods for inhibiting proliferation and inducing cell death in a population of cancer cells by (i) increasing the amount of the differentiation associated protein, *MDA*-7 and (ii) decreasing *RAS* activity within the population. A "population" is defined herein as comprising at least two cells. Non-limiting examples of populations include a solid tumor, an infiltrating tumor, cancer cells disseminated within a subject (*e.g.*, as would be present in a subject with metastatic disease), and a cell culture. To practice the full scope of the invention, the amount of *MDA*-7 may be increased intracellularly and/or extracellularly and an increase in *MDA*-7 protein and/or a decrease in *RAS* activity need not be achieved in all the cells of the population.

In particular embodiments, the present invention relates to methods for inhibiting proliferation and/or inducing cell death of a cancer cell by (i) increasing the amount of the differentiation associated protein, *MDA*-7 and (ii) decreasing *RAS* activity in the cancer cell.

The cancer cell in the foregoing methods may be a pancreatic cancer cell or other suitable target cell, as described in the foregoing section.

The amount of *MDA*-7 may be increased by introducing, into a cancer cell, a nucleic acid encoding *mda*-7 in expressible form, or by administering, to the cancer cell and/or to the population of cancer cells, *MDA*-7 protein, as set forth in section 5.1, *above*, such that an amount of *MDA*-7 protein is present which is effective, in the presence of decreased *RAS* activity, in decreasing cell proliferation and inducing cell death.

For example, and not by way of limitation, where *mda*-7 is to be introduced into a cancer cell via a viral vector, the amount of virus to which the cell is exposed may be between about 1-1000 pfu/cell, and preferably between about 100-250 pfu/cell. In the working example set forth in Section 6, *below*, 100 pfu/cell of *mda*-7 comprised in a replication defective adenovirus vector was used.

Where *MDA*-7 protein is to be administered to a cell, in specific non-limiting embodiments of the invention, the concentration of *MDA*-7 to which the cell is exposed may be between about 1-100 ng/ml .

The amount of *RAS* activity may be decreased by administering, to the cancer cell or cancer cell population, an effective amount of an anti-*RAS* agent, which may be, for example, an antisense molecule, a ribozyme, a precursor of a triple helix, a small molecule with a defined structure (see Section 5.4, above) or a farnesyl transferase inhibitor or an agent that acts at some other point of the *RAS* pathway, for example, but not limited to, an antagonist of the EGF receptor or an inhibitor of *RAF*, MAPK kinase, or PI3 kinase . In preferred embodiments of the invention, an antisense molecule, particularly an antisense oligonucleotide, and most preferably an antisense oligonucleotide having phosphorothioate linkages is used. In particular embodiments, where a specific mutation of *ras* in the cancer cell to be treated has been identified, a mutation specific oligonucleotide may be used.

In preferred non-limiting embodiments of the invention, a phosphorothioate antisense oligonucleotide 5'-CACAAGTTTATATTCAGT -3' (SEQ ID NO:15), or an oligonucleotide comprising said nucleotide or hybridizable to the complement of said nucleotide under stringent conditions, may be used.

In specific, non-limiting embodiments, the concentration of oligonucleotide to which a cancer cell is exposed may be between about 0.1 - 10

micromolar and preferably between about 0.25 -0.75 micromolar. In the experiments described in Section 6, below, a concentration range of 0.1-5.0 micromolar was used.

An "effective amount" of a combination of agents that increase *MDA*-7 and decrease *RAS* activity is an amount of agents which result in a decrease in cancer cell proliferation and an increase in cancer cell death. The amount of each agent may not, and probably is not, effective in the absence of the other. Preferably, but not by way of limitation, the decrease in cell proliferation is by at least 25 percent, and the increase in cell death is by at least about 25 percent, relative to a control cancer cell not exposed to either agent.

The present invention and the foregoing methods may be applied to the treatment of a subject with a cancer. The subject may be a human or a non-human mammalian subject, and the cancer may be comprised of pancreatic cancer cells or other suitable target cells, as defined in the foregoing section. In particular non-limiting embodiments, the present invention provides for a method for treating a subject having pancreatic cancer, comprising, administering, to the subject, amounts of agents which are effective, in combination, in (i) increasing the amount of the differentiation associated protein, *MDA*-7 and (ii) decreasing *RAS* activity in cells of the pancreatic cancer. In specific non-limiting embodiments, the subject having pancreatic cancer is treated by administering, to the subject, (a) a viral vector comprising an *mda*-7 gene in expressible form; and (b) an antisense *ras* oligonucleotide, in amounts which are effective, in combination, in (i) increasing the amount of the differentiation associated protein, *MDA*-7 and (ii) decreasing *RAS* activity in cells of the pancreatic cancer.

The subject may be administered a therapeutically effective amount of a combination of *MDA*-7 increasing and *RAS* activity decreasing agents by a suitable route, including intra-tumor instillation, intravenous, intraarterial, intrathecal, intramuscular, intradermal, subcutaneous, etc.. A therapeutically effective amount of these agents produces one or more of the following results: a decrease in tumor mass, a decrease in cancer cell number, a decrease in serum tumor marker, a decrease in tumor metastasis, a decreased rate of tumor growth, improved clinical symptoms, and/or increased patient survival. The cancer may be first treated surgically to de-bulk the tumor mass, if appropriate.

Where a viral vector is used to deliver either *mda*-7 and/or antisense *ras*-encoding nucleic acid sequences, the amount of vector administered to the

subject, in preferred embodiments, is between about 10^9 and 10^{13} pfu. Where a replication defective viral vector is being used to either deliver *mda*-7 or antisense *ras* encoding nucleic acid, it is preferable to deliver such vector directly into or around the cancer cells, for example, by intra-tumor injection or by instillation into the tumor bed following surgical resection

Where antisense oligonucleotides are being administered, the effective dosage may vary among formulations. Optimum dosages may be determined based on the EC_{50}s determined *in vitro*, in cell culture, and/or *in vivo*, in animal models. For example, but not by way of limitation, the dosage may range from 0.01 micrograms to 10 mg per kg of body weight.

The present invention also may be applied to the treatment of metastatic cancer in a subject. Metastatic cancer is defined herein as the occurrence of one or more cancer cell at a site which is not directly contiguous with a primary tumor (*e.g.*, cancer cells from a non-lymphoid tumor in a lymph node receiving lymphatic drainage from the site of the primary tumor, cancer cells identified in the lung, brain, bone, or liver where the primary cancer does not originate in respectively lung, brain, bone or liver). The definition of "metastatic cancer" also encompasses instances where a primary tumor cannot be identified, in which case there is a histological determination that a cancer cell or group of cancer cells did not originate in the tissue where it (they) has (have) been detected.

In a subset of such embodiments, the present invention provides a means for treating metastatic cancer in a subject which addresses the issue that it may be difficult to provide a sufficient number of viral vectors carrying the *mda*-7 gene to achieve infection of cancer cells in distant metastases. This problem may be addressed by infecting a population of cells in the subject with an *mda*-7 bearing viral vector, and thereby providing distant cancer cells in the subject with *MDA*-7 released or secreted by the infected population. The effectiveness of such released/secreted *MDA*-7 at inhibiting proliferation of cancer cells is supported by data provided in Section 8, below. The distant cancer cells may be further treated by antisense *ras* in the form of oligonucleotides (*e.g.*, phosphorothioate linked oligonucleotides). Non-limiting examples of end points that may be examined as a means of establishing efficacy of treatment include decreases in the number and size of metastatic tumors, decreases in their rate of growth, and/or increases in the length of survival of the

subject. One skilled in the art will recognize that many other objective measures of morbidity and/or mortality also may be examined to assess therapeutic efficacy.

In particular embodiments, treatment of metastatic disease may be achieved through administering, to the subject, amounts of agents which are effective, in combination, in (i) increasing the amount of the differentiation associated protein *MDA*-7 in blood or other extracellular fluids and (ii) decreasing *RAS* activity in target cells residing in both the primary tumor site and in metastatic sites. In specific non-limiting embodiments, the subject having metastatic cancer is treated by administering, to the subject, (a) a viral vector comprising an *mda*-7 gene in an expressible form; and (b) an antisense *ras* oligonucleotide, in amounts which are effective, in combination, in (i) increasing the amount of the differentiation associated protein *MDA*-7 in blood or other extracellular fluids and (ii) decreasing *RAS* activity in cells of the both the primary tumor and metastatic tumor locations. A preferred form of this embodiment may comprise the combined administration of (i) an anti-*ras* phosphorothioate antisense oligonucleotide, such as that represented by SEQ ID NO: 15 or other nucleotides sequences hybridizable to the complement of said nucleotide sequence under stringent conditions, at doses capable of achieving concentrations of the oligonucleotide between 0.1 and 50 micromolar in the fluids surrounding the cancer cell and (ii) a viral vector encoding *mda*-7, after which the MDA-7 protein is synthesized in and released from the virally-transduced cells into the blood or other extracellular fluids so that MDA-7 concentrations of between 1 to 100 ng/ml could be attained in the fluids surrounding the target cells. In specific non-limiting embodiments of the invention, a viral vector carrying a *mda*-7 gene may be introduced into an identified population of cells, for example hepatocytes, or cells of the primary tumor.

In related embodiments based on the discovery that released/secreted *MDA*-7 has antiproliferative activity, the present invention provides for methods of treating cancer cells which are responsive to *MDA*-7 without requiring a decrease in *RAS* activity, comprising exposing said cancer cells to extracellular *MDA*-7 protein at a level effective in inhibiting the proliferation of said cells. Such extracellular protein may be provided by administration of protein or by introducing an *mda*-7 gene into cells of the subject (which may be any type or types of non-malignant or malignant cells of the subject, *e.g.*, normal hepatocytes, fibroblasts, muscle cells, cancer cells, combinations of different cell types, etc.) such that cells release/secrete *MDA*-7 which

may then inhibit cancer cell proliferation/survival, even at distant cites. Cancer cells susceptible to *MDA*-7 antiproliferative effects include, but are not limited to, melanoma cells, glioblastoma multiforme cells, osteosarcoma cells, breast cancer cells, cervical cancer cells, colon cancer cells, lung cancer cells, nasopharynx cancer cells, ovarian cancer cells, and prostate cancer cells. A growth suppressive effect of culture medium of Ad.*mda*-7-infected hepatocytes (providing extracellular *MDA*-7) on human prostate cancer cells has been observed (see Section 8, below).

The foregoing treatments may be administered in conjunction with other surgical and non-surgical therapeutic modalities, including chemotherapy, gene therapy, immunotherapy, and radiation therapy.

6. EXAMPLE: A COMBINATORIAL APPROACH FOR SELECTIVELY INDUCING PROGRAMMED CELL DEATH IN HUMAN PANCREATIC CANCER CELLS

6.1. MATERIALS AND METHODS

Cell lines, culture conditions and growth assays. The AsPC-1, BxPC-3, MIA PaCa-2 and PANC-1 human pancreatic carcinoma cell lines (obtained from the American Type Culture Collection) were grown in RPMI 1640 containing 10 percent fetal bovine serum ("FBS") at 37°C in a 95 percent air 5 percent carbon dioxide humidified incubator. Cell growth and viable cell numbers were monitored by hemocytometer and MTT staining as described in (Lebedeva et al., 2000, Cancer Res. 60:6052-6060).

Animal studies. Tumorigenicity assays were performed as described in Madireddi et al., 2000, Adv. Exptl. Med. Biol. 465:239-261 and Su et al., 1998, Proc. Natl. Acad. Sci. U.S.A. 95:14400-14405. Briefly, MIA PaCa-2 cells were untreated or infected with 100pfu/cell of Ad.*vec* or Ad.*mda*-7 and then untransfected or transfected with an expression vector containing a 346 nt k-*ras* gene fragment (nt 172 to nt 517) cloned in a sense or AS orientation and 1 x 10^6 cells were mixed with Matrigel and injected 48 hours later subcutaneously into athymic nude mice. Animals were monitored for tumor formation and tumor volume was determined as described in Madireddi et al., 2000, Adv. Exptl. Med. Biol. 465:239-261.

Viral construction, purification and infectivity assays. The replication defective Ad.*mda*-7 was created in two steps. In the first step, the *mda*-7 gene was cloned into a modified adenovirus expression vector Ad.CMV (Falck-Pedersen et al.,

1994, Mol. Pharmacol. 45:684-689). This contains, in order, the first 355 bp from the left end of the Ad genome, the cytomegalovirus immediate early promoter, DNA encoding splice donor and acceptor sites, cloning sites for the insertion of the *mda*-7 expression cassette, DNA encoding a polyA signal sequence from the β globin gene, and approximately 3 kilobase pairs of adenovirus sequence extending from within the E1B coding region. This arrangement allows high level expression of the cloned sequence by the cytomegalovirus immediate early gene promoter, and appropriate RNA processing. In the second step, the recombinant virus was created *in vivo* by 293 cells (Graham et al., 1977, J. Gen. Virol. 36:59-72) by homologous recombination between the *mda*-7 containing vector and plasmid JM17. Following transfection of the two plasmids, infectious virus was recovered, the genomes were analyzed to confirm the recombinant structure, and then virus was plaque purified and titrated (Volkert and Young, 1983, Virology 125:175-193).

Phosphorothioate oligonucleotides. Eighteen-base phosphorothioate oligonucleotides ("PS ODN") were synthesized and purified by HPLC (Lebedeva et al., 2000, Cancer Res. 60:6052-6060). The antisense *ras* phosphorothioate antisense oligonucleotide ("AS K-*ras* PS ODN") CACAAGTTTATATTCAGT (SEQ ID NO:15) was synthesized and is complementary to wild type K-*ras* nucleotides 196-213 (adjacent the start codon). Based on previous studies (Sakakura et al., 1995, Anti-Cancer Drugs 6:553-561), mismatched ("MM") K-*ras* PS ODN CACTTGCAAATATTCAGT (SEQ ID NO:16) and scrambled ("SC") K-*ras* PS ODN ACTAGCTATACTAGCTAT (SEQ ID NO:17) to the same region (nt 196-213) were also synthesized.

RNA isolation and Northern blot analysis. Total RNA was isolated by a guanidinium/phenol procedure and Northern blots were performed as described in Jiang and Fisher, 1993, Mol. Cell. Different. 1:285-299 and Jiang et al., 1995, Oncogene 11:2477-2486.

DNA extraction, fragmentation assays, FACS analysis and Annexin V, PI and DAPI staining. DNA was extracted and fragmentation assays were performed as described in Su et al., 1995, Anticancer Res. 15:1841-1848 three days after a single or combination treatment protocol. FACS analysis and annexin V and PI staining were performed using previously described methods (Su et al., 1998, Proc. Natl. Acad. Sci. U.S.A. 95:14400-14405; Lebedeva et al., 2000, Cancer Res. 60:6052-6060;

Su et al., 1995, Anticancer Res. 15:1841-1848; Martin et al., 1995, J. Exp. Med.182:1545-1556).

Western blotting. Cell extracts in RIPA buffer were prepared and equal concentrations of proteins were evaluated for *MDA-7*, *BCL-2*, *BAX* and *EF-1α* protein levels by Western blotting as described in Kita et al., 1999, Intl. J. Cancer 80:553-558; Lebedeva et al., 2000, Cancer Res. 60:6052-6060 and Su et al., 1995, Intl. J. Oncol. 7:1279-1284.

6.2. RESULTS AND DISCUSSION

The combination of Ad.*mda-7* and AS K-*ras* PS ODN synergistically suppressed growth in mut K-*ras* expressing human pancreatic carcinoma cells. *Mda-7* is a broad-spectrum cancer-specific growth -suppressing gene, which displays no apparent harmful effects in normal cells (Jiang et al., 1995, Oncogene 11:2477-2486; Jiang et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:9160-9165; Madireddi et al., 2000, Adv. Exptl. Med. Biol. 465:239-261; Su et al., 1998, Proc. Natl. Acad. Sci. U.S.A. 95:14400-14405; Saeki et al., 2000, Gene Ther. 7:2051-2057; Mhashilkar et al., 2001, Mol. Med. 7:271-282). Infection of a diverse group of human cancers with Ad.*mda-7*, including melanoma, glioblastoma multiforme and osteosarcoma, and carcinomas of the breast, cervix, colon, endometrium, lung and prostate, results in growth suppression and hypodiploidy, a cellular change frequently associated with apoptosis (Jiang et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:9160-9165; Madireddi et al., 2000, Adv. Exptl. Med. Biol. 465:239-261; Su et al., 1998, Proc. Natl. Acad. Sci. U.S.A. 95:14400-14405; Saeki et al., 2000, Gene Ther. 7:2051-2057; Mhashilkar et al., 2001, Mol. Med. 7:271-282). In a detailed study with several breast carcinoma cell lines, the ability of Ad-*mda-7* to induce growth suppression was found to be independent of *p53*-status and to correlate with induction of apoptosis, as monitored by DNA nucleosomal laddering, the TUNEL reaction and Annexin V staining (Madireddi et al., 2000, Adv. Exptl. Med. Biol. 465:239-261; Su et al., 1998, Proc. Natl. Acad. Sci. U.S.A. 95:14400-14405; Mhashilkar et al., 2001, Mol. Med. 7:271-282). In contrast, growth was minimally affected and no induction of apoptosis was apparent in early passage normal mammary epithelial cells or the normal breast epithelial cell line, HBL-100, after infection with Ad.*mda-7*.

When evaluating the effect of *mda-7* on diverse cancer subtypes it was readily apparent that pancreatic carcinoma cells are inherently resistant to ectopic

expression of *mda*-7. Infection of mutated or wild type K-*ras* expressing pancreatic carcinoma cells with 100 pfu/cell of Ad. *mda*-7 or Ad.*vec* (the Ad construct lacking the *mda*-7 gene insert) did not significantly affect growth and no selective induction of apoptosis was evident (see FIGURE 5). A dose-dependent growth inhibitory effect was apparent when the different pancreatic carcinoma cells were treated with 0.1 to 5 micromolar antisense K-*ras* phosphorothioate oligonucleotide (AS K-*ras* PS ODN), with a maximum inhibition of ~10 to ~30 percent depending on the cell type when treated for 3 or 4 days with 5 micromolar AS K-*ras* PS ODN (FIGURE 5). Growth of BxPC-3, which carries a wild type K-*ras* gene, was inhibited the least by the K-*ras* PS ODN. Selectivity of the AS K-*ras* PS ODN was suggested by the fact that treatment with either scrambled ("SC") or mismatched ("MM") PS ODN resulted in significantly less growth suppression than treatment with the AS K-*ras* PS ODN (see FIGURE 5). These studies document that a single application of Ad.*mda*-7 or AS K-*ras* PS ODN to mutated or wild type K-*ras* pancreatic carcinoma cell lines can induce variable degrees of growth suppression. However, in all cases growth suppression was transient and cells survived the single treatment and continued to proliferated, even when initially exposed to 5 micromolar PS ODN.

When mutated K-*ras* pancreatic carcinoma cells were infected with Ad.*mda*-7 and then treated with 0.1 to 5.0 micromolar AS K-*ras* PS ODN, but not SC or MM PS ODN, a profound synergistic growth inhibitory effect and a decrease in cell survival were evident (FIGURES 5 and 6M-O). In contrast, no synergistic growth inhibition or decrease in cell viability was detected in wt K-*ras* BxPC-3 cells (FIGURES 5 and 6P). Additionally, no effect on growth or viability was apparent with any of the treatments in early passage normal human prostate epithelial cells or when pancreatic cancer cells were infected with an adenovirus expressing luciferase or β-galactosidase and then treated with AS K-*ras* ODN. These results document an anti-survival effect of the combination of *mda*-7 and AS K-*ras* PS ODN in mutated K-*ras* pancreatic carcinoma cells, but not in wild type K-*ras* pancreatic cancer cells or normal epithelial cells.

A previous study reported that AS K-*ras* PS ODN that target specific point mutations in K-*ras* codon 12 can reduce growth in mutant pancreatic carcinoma cell lines, but not in wild type K-*ras* BxPC-3 cells (Kita et al., 1999, Intl. J. Cancer 80:553-558). This effect was greater using appropriate mutation-mismatched AS PS ODN versus mutation-mismatched PS ODN. Effects on growth, although less, were

also apparent when using AS PS ODN that did not correspond precisely to the mutation in K-*ras* codon 12 of the particular pancreatic carcinoma analyzed. This observation supports numerous previous studies indicating that AS PS ODN can induce both specific and apparently non-specific effects in target cells (Stein and Cheng, 1993, Science 261:1004-1012; Stein, 1996, Trends Biotechnol. 14:147-149; Pawlak et al., 2000, Cancer Treat. Rev. 26:333-35030-32).

In the present study, AS K-*ras* PS ODN were designed to interact with the AUG start codon of the K-*ras* gene. Treatment of both mutated and wild-type K-*ras* expressing pancreatic carcinoma cells with AS K-*ras* PS ODN, but not MM or SC PS ODN, reduced K-*ras* p21 protein levels in both mutated and wild-type K-*ras* cells by greater than 80 percent within 24 hours (FIGURE 7). This effect was observed with and without Ad.*vec* or Ad.*mda*-7 infection, which did not consistently cause a further alteration in K-*ras* levels (FIGURE 7). Moreover, the growth inhibitory effect of the AS K-*ras* PS ODN was greater in the three mutated K-*ras* pancreatic carcinoma cells than in the wild type K-*ras* BxPC-3 cell line (FIGURE 5). As observed in the study of Kita et al. (1999, Intl. Journal of Cancer 80:553-558), growth inhibition induced by AS K-*ras* PS ODN, either point or start codon specific (as in this example), exceeded that observed using SC or MM PS ODN. In addition, no synergistic growth inhibitory effects or decreases in cell survival were apparent in Ad.*mda*-7 infected pancreatic carcinoma cells that were subsequently treated with SC or MM PS ODN. These results confirm a profound synergistic growth inhibitory effect specifically in mutated K-*ras* pancreatic carcinoma cells after infection with Ad.*mda*-7 and treatment with AS K-*ras* PS ODN.

Plasma membrane associated small molecular weight GTP-binding proteins are frequently utilized by cells in the process of signal transduction from the inner leaflet of the plasma membrane to the cytosol. The prototypical small molecular weight family of GTP-binding proteins is the *ras* gene family (Kolch, 2000, Biochem. J. 351:289-305). Based on the observation that K-*ras* mutations appear in atypical hyperplastic ducts that surround the ductal-like cancer cells (Lemoine et al., 1992, Gastroenterol. 102:230-236), it is currently believed that K-*ras* mutations represent a very early event in pancreatic carcinogenesis. The resulting K-*ras* mutation induces a conformational change in the molecule and a concomitant maintenance of *RAS* activation by decreasing hydrolysis of GTP to GDP (Kolch, 2000, Biochem. J. 351:289-305; Reuther and Der, 2000, Curr. Opin. Cell Biol. 12:157-165). When

activated, K-*RAS* can signal into the cytosol via multiple downstream signaling pathways such as the classical MAPK pathway; the P13 kinase pathway; and the JNK pathway, to induce a plethora of cellular changes, including enhanced proliferation (Dent et al., 1992, Science 257:1404-1407; Gire et al., 2000, Oncogene 19:2269-2276; Almeida et al., 2000, J. Cell Biol. 149:741-754). In these contexts, blocking K-*RAS* expression may alter downstream pathway activities in mutated K-*ras* pancreatic cancer cells, rendering these cells sensitive to *MDA*-7 induction of growth suppression and effects on cell viability.

Infection of mutated K-*ras* pancreatic carcinoma cells with Ad. *mda*-7 followed by transfection with as AS K-*ras* expression vector inhibited growth *in vitro* and tumorigenesis *in vivo* in nude mice. As an additional approach to inhibiting K-*ras* expression, a K-*ras* gene fragment of 346 nucleotides (extending from nucleotide 172 to nucleotide 517), that was previously shown to inhibit pancreatic cancer cell growth *in vitro* and *in vivo* when used in an antisense orientation (Aoki et al., 1995, Cancer Res. 55:3810-3816; Aoki et al., 1997, Mol. Carcinogen. 20:251-258), was isolated by PCR from BxPC-3 cells and cloned into a pcDNA3.1 (neomycin resistance) expression vector. This expression vector was then tested for effects on pancreatic carcinoma cells, when used alone or in combination with Ad.*mda*-7. As can be seen in FIGURE 8 (upper row of culture plates), infection of MIA PaCA-2 cells with Ad.*vec* alone or in combination with AS K-*ras* PS ODN did not significantly alter colony formation. Similarly, infection of MIA PaCa-2 cells with Ad.*mda*-7 also did not modify cloning efficiency in monolayer culture. In contrast, the combination of Ad.*mda*-7 with AS K-*ras* PS ODN dramatically inhibited colony formation (confirming previous studies using cell counting by hemocytometer and MTT staining). To test the effect of the AS K-*ras* plasmid in combination with Ad.*mda*-7 on pancreatic carcinoma cell growth, MIA PaCa-2 cells were infected with Ad.*vec* or Ad.*mda*-7 and transfected with a control or the AS K-*ras* plasmid and G418-resistant colony formation was determined. As can be seen in FIGURE 8 (lower row of culture plates), a dramatic suppression in growth was observed only in MIA PaCa-2 cells infected with Ad.*mda*-7 and transfected with the As K-*ras* plasmid. Quantitatively similar growth inhibitory results were obtained when the same protocols were used with AsPC-1 or PANC-1 mutated K-*ras* pancreatic carcinoma cells, but not with wild type K-*ras* BxPC-3 cells. These results indicate that both AS

PS ODN and antisense K-*ras* expression by plasmid transfer can synergize with *mda*-7 to inhibit mutated K-*ras* pancreatic carcinoma cell growth.

MIA PaCa-2 cells form tumors in athymic nude mice with a short latency time. Transfection with an AS K-*ras* plasmid or infection with Ad.*mda*-7 resulted in rapidly growing tumors in 80 percent of animals (3 independent experiments, n=26). Similarly, infection with Ad.*vec*, a plasmid lacking the gene inserts, or transfection with a plasmid construct containing a 346 nt K-*ras* gene fragment cloned in a sense orientation did not significantly inhibit tumor formation (76 percent tumors; n=17; 3 independent experiments).

In contrast, a remarkable complete suppression in tumor formation was apparent only when MIA PaCa-2 cells were infected with Ad.*mda*-7 and then transfected with the AS K-*ras* plasmid prior to being injected into athymic nude mice (no tumors formed in 13 animals; 3 independent studies). These findings document that in mutated K-*ras* pancreatic cancer cells, infection with Ad. *mda*-7 combined with targeting the K-*ras* gene for inhibition in a small subset of cells by means of transfection with an AS K-*ras* expression plasmid eliminated *in vivo* tumor formation in nude mice. Since transfection is an inherently inefficient means of introducing genes into target cells, it is possible that cells receiving the combination treatment release factor(s) that sensitize adjacent tumor cells containing *mda*-7 to lose viability, thereby preventing tumor formation.

The combination of Ad.*mda*-7 and AS K-*ras* PS ODN induced apoptosis selectively in mutated K-*ras* expressing human pancreatic carcinoma cells. The mechanism by which *MDA*-7 selectively decreases colony formation and growth in human cancer cells involves induction of apoptosis (Madireddi et al., 2000, Adv. Exptl. Med. Biol. 465:239-261; Su et al., 1998, Proc. Natl. Acad. Sci. U.S.A. 95:14400-14405; Saeki et al., 2000, Gene Ther. 7:2051-2057; Mhashilkar et al., 2001, Mol. Med. 7:271-282). To determine if the combination treatment of K-*ras* mutated pancreatic cancer cells decreases cell survival by induction of apoptosis, a number of assays typically used to monitor programmed cell death were performed. In many cell types, induction of apoptosis is associated with DNA degradation, which can be monitored by generation of nucleosomal DNA ladders (Su et al., 1998, Proc. Natl. Acad. Sci. U.S.A. 95:14400-14405; Reed, 2000, Am. J. Pathol. 157:1415-1430; Green and Reed, 1998, Science 281:1309-1312). As can be seen in FIGURE 9, treatment of mutated K-*ras*- expressing pancreatic carcinoma cells, but not wild-type K-ras

expressing BxPC-3, with Ad.*mda*-7 plus As K-*ras* PS ODN resulted in DNA fragmentation. The specificity of this effect was further documented by the lack of nucleosomal DNA ladders in pancreatic cancer cells infected with Ad.*mda*-7 or treated with 5.0 μM AS K-*ras* PS ODN alone or in cells treated with the combination of Ad. *mda*-7 with 5.0 μM MM K-*ras* PS ODN. Confirmation of induction of apoptosis by combination treatment in the three mutated K-*ras* pancreatic carcinoma cells was verified by DAPI and by propidium iodide staining, increases in the number of hypodiploid cells and Annexin V staining by FACS analysis. These results confirm that the combination of Ad.*mda*-7 and AS K-*ras* PS ODN decreased viability in mutated K-*ras* expressing pancreatic carcinoma cells by inducing apoptosis.

MDA-7 protein was present in mutated K-*ras* expressing human pancreatic carcinoma cells following infection with Ad.*mda*-7 and treatment with AS K-*ras* PS ODN. The reason that pancreatic carcinoma cells are resistant to *mda*-7 and the mechanism by which the combination of Ad.*mda*-7 and AS K-*ras* PS ODN sensitizes specific pancreatic carcinoma cells to *mda*-7 induction of growth suppression and apoptosis is not known. One hypothesis is that the mutated K-*ras* protein, or biochemical pathways modified by this protein, prevents synthesis, processing and/or secretion of *MDA*-7 protein following infection with Ad.*mda*-7. This possibility was tested by determining the effect of various treatment protocols on intracellular *MDA*-7 protein levels in the different pancreatic carcinoma cell lines (FIGURE 10A-D). No *MDA*-7 protein was detected in cell lysates from the four different pancreatic carcinomas 24 hours after infection with Ad.*mda*-7 alone or in combination with MM or SC PS ODN. This occurred despite the production of *mda*-7 mRNA in all four pancreatic cancer cell lines following infection with Ad.*mda*-7 (FIGURE 11). In contrast, *MDA*-7 protein was readily detected in the three K-*ras* mutated pancreatic carcinoma cell lines after infection with Ad. *mda*-7 and treatment with AS K-*ras* PS ODN (FIGURE 10A-C). In the case of wild-type K-*ras* expressing BxPC-3, *MDA*-7 protein was not detected (FIGURE 10D). These results suggest that mutated K-*ras* may negatively affect *MDA*-7 protein processing in mutated K-*ras*-pancreatic cancer cells. The absence of *MDA*-7 protein, using similar protocols, in BxPC-3 cells suggests that other pathways may be operational that modify expression and/or retention of *MDA*-7 protein in these pancreatic carcinoma cells. Since apoptosis only occurs in K-*ras* mutated pancreatic cancer cells treated with the combination of *mda*- 7 and antisense *ras*, these studies support a potential correlation

between presence/retention of *MDA*-7 protein and induction of growth suppression and programmed cell death in pancreatic carcinoma cells.

The combination of Ad.*mda*-7 and AS K-*ras* PS ODN alters the levels of apoptosis-associated proteins. Previous studies indicate that infection of diverse cancer cells with Ad.*mda*-7 results in apoptosis, and in the majority of cases this process is associated with up-regulation of *BAX* protein and changes in the ratio of *BAX* to *BCL-2* protein (Madireddi et al., 2000, Adv. Exptl. Med. Biol. 465:239-261; Su et al., 1998, Proc. Natl. Acad. Sci. U.S.A. 95:14400-14405; Saeki et al., 2000, Gene Ther. 7:2051-2057; Mhashilkar et al., 2001, Mol. Med. 7:271-28218-21). However, the ability of Ad.*mda*-7 to induce apoptosis in specific cancer cells, such as DU-145 human prostate carcinoma cells which do not produce *BAX* protein (Rampino et al., 1997, Science 275:967-969), indicates that *mda*-7 can also mediate programmed cell death in certain cancer cells by a *BAX*-independent pathway. Based on these considerations and the presence of *MDA*-7 protein specifically in combination treated mutated K-*ras* pancreatic carcinoma cells, experiments were performed to determine the levels of *BAX* and *BCL-2* proteins in treated cells. When analyzed 3 days after combination treatment, in which the majority of K-*ras* mutated cells were apoptotic, the levels of *BAX* protein were elevated in PACN-1, MIA PaCa-2 and AsPC-1 cells, ~7.5-, ~3- and ~10-fold, respectively, but not in BxPC-3 cells (FIGURE 12). Moreover, the levels of *BCL-2* protein were significantly reduced in PANC-1(~8-fold) and MIA PaCa-2 (~13.5-fold) cells, marginally reduced in AsPC-1 cells (~1.2-fold) and remained unchanged in BxPC-3 cells (FIGURE 12). These results are consistent with involvement of *BAX* protein and changes in the ratio of *BAX* to *BCL-2* proteins in inducing apoptosis in combination treated pancreatic carcinoma cells.

7. EXAMPLE: ANTISENSE *RAS* NUCLEIC ACIDS HAVING 346 OR 631 NUCLEOTIDES, IN COMBINATION WITH *mda*-7, INHIBIT COLONY FORMATION OF PANCREATIC CANCER CELLS

7.1. MATERIALS AND METHODS

MIA PaCa-2 cells were infected with 100 pfu/cell of Ad.*mda*-7, and then were transfected with 10 micrograms of a wild-type K-*ras* gene fragment of 346 nucleotides (nucleotides 172 to 517 of the K-*ras* cDNA) or 631 nucleotides

(nucleotides 172 to 802 of the K-*ras* cDNA), cloned in the antisense orientation in the pcDNA3.1 expression vector, in the presence of 10 micromolar lipofectamine. As controls, some cells were infected with 100 pfu/cell of AD.*mda*-7 and then transfected with 10 micrograms of empty pcDNA3.1 vector, and other cells were not adenovirus-infected, but were only transfected with the empty pcDNA3.1 vector. Twelve hours after transfection the cells were seeded at various cell densities and selected in G418-containing medium and colony formation was assessed after 2-3 weeks. Analogous experiments were performed using PANC-1 and AsPC-1 cells.

7.2. RESULTS

The results of the foregoing experiments using MIA PaCa-2 cells are shown in bar graph format in FIGURE 13. Colony formation was inhibited slightly, if at all, by *mda*-7 alone or either transfection of 346 or 631 nucleotide *ras* antisense sequences without Ad.*mda*-7 infection. However, infection with Ad.*mda*.7 combined with transfection with either the 346 or 631 nucleotide antisense *ras*-encoding plasmid resulted in substantial inhibition of colony formation, indicating that *ras* antisense nucleotides of diverse sizes can exert effective inhibition of *RAS* activity. Qualitatively similar results were observed in PANC-1 and AsPC-1 cells.

8. EXAMPLE: SECRETION OF BIOLOGICALLY FUNCTIONAL *MDA*-7 FOLLOWING INFECTION OF PRIMARY RAT HEPATOCYTES WITH AD.*mda*-7

8.1. OVERVIEW

Systemic infection with adenovirus vectors invariably results in high levels of infection in the liver (Huard et al., 1995, Gene Ther. 2(2):107-115; Morral et al., 1999, Proc. Natl. Acad. Sci. U.S.A. 96:12816-12821). In specific instances this interaction can result in toxicity (Somia and Verma, 2000, Nat. Rev. Genet. 1(2):91-99). Previous studies have documented that *mda*-7 is not toxic to a spectrum of normal human cell types, including skin and lung fibroblasts, breast and prostate epithelial cells, endothelial cells, and melanocytes (Madireddi et al., 2000, Adv. Exp. Med. Biol. 465:239-261; Saeki et al., 2000, Gene Ther. 7:2051-2057; Mhashilkar et al., 2001, Mol. Med. 7:271-282). In contrast, *mda*-7 selectively induces growth suppression and programmed cell death (apoptosis) in a diverse spectrum of human tumor cells, including melanoma, glioblastoma multiforme, osteosarcoma and

carcinomas of the breast, cervix, colon, lung, nasopharynx, ovary and prostate (Madireddi et al., 2000, Adv. Exp. Med. Biol. 465:239-261; Saeki et al., 2000, Gene Ther. 7:2051-2057; Mhashilkar et al., 2001, Mol. Med. 7:271-282). Pancreatic carcinoma cells, however, are refractive to Ad.*mda*-7, unless infection is combined with approaches that decrease *RAS* activity. The experiments described in this section were performed to (1) determine if infection of primary hepatocytes with Ad.*mda*-7 induces toxicity; and (2) determine if infection of primary hepatocytes with AD.*mda*-7 results in the production of biologically active *MDA*-7 protein inside cells and in medium used to grow the infected hepatocytes.

8.2. MATERIALS AND METHODS

Preparation of primary rat hepatocytes and preparation of conditioned medium and cell lysates. Primary rat hepatocytes were isolated using the two stage collagenase perfusion technique as described in Park et al., 2000, Mol. Biol. Cell. 11:2915-2932. Four hours after attachment, cells were infected at a multiplicity of infection of 30 with either a null adenovirus vector (Ad.*vec*) or Ad.*mda*-7 (Su et al., 1998, Proc. Natl. Acad. Sci. U.S.A. 95:14400-14405). Cells were gently rocked during this process to promote viral adsorption and infection. Eighty-four hours after infection, the media was removed from the cells and used for further experimentation, as described below. Infected hepatocytes were scraped into the same volume of sterile Dulbecco's Modified Eagle's Medium ("DMEM") that was used during cell culture, lysed by a single freeze-thaw cycle at -20°C with triturating on thawing through a P1000 pipette tip, and then the cellular debris was removed by centrifugation and media containing the cellular extract was decanted. The media was used for further experimentation, also as described below.

Assay of Ad.*mda*-7 and Ad.*vec*-infected hepatocyte conditioned medium and lysed cells for biological activity toward human pancreatic cancer cells. MIA PaCa-2 cells were seeded at 1 x 10^6/10 cm plate. The next day, cells were allocated into the following groups and treated as follows: (1) untreated control ; (2) Ad.*mda*-7 infected (using 100 pfu/cell); (3) transfected with AS K-*ras* phosphorothioate oligonucleotides having SEQ ID NO:15 at a concentration of 0.5 micromolar in the presence of 10 microgram per milliliter of lipofectamine; or infected with Ad.*mda*-7 and transfected with AS K-*ras* phosphorothioate oligonucleotide, using the same conditions as for groups (2) and (3). The next day the

cells from the various groups were resuspended using trypsin/versene and replated in duplicate at 1 x 10^5/35 mm plate. After cell attachment had occurred (about 6 hours), the growth medium was removed and replaced with either Ad.*mda*-7-infected hepatocyte lysate or culture supernatant (diluted 1:1 with DMEM + 10% fetal bovine serum), prepared as described above, or, for controls, fresh growth medium. Cell numbers were determined every other day over an 8 day period.

8.3. RESULTS

The results are depicted in FIGURE 14A-B, for control cells treated with either *mda*-7 infected hepatocyte lysate (FIGURE 14A) or supernatant (FIGURE 14B); FIGURE 15A-B for Ad.*mda*-7 infected MIA PaCa-2 cells treated with either *mda*-7 infected hepatocyte lysate (FIGURE 15A) or supernatant (FIGURE 15B); and FIGURE 16A-B for AS K-*ras* oligonucleotide transfected MIA PaCa-2 cells treated with either *mda*-7 infected hepatocyte lysate (FIGURE 16A) or supernatant (FIGURE 16B). FIGURES 15A-B and 16A-B also depict the cell numbers for MIA PaCa-2 cells which had been both infected with Ad.*mda*-7 and transfected with AS K-*ras* oligonucleotide.

Infection of primary rat hepatocytes with Ad.*mda*-7 (30 pfu/cell) did not induce a toxic effect in primary liver cells. The infected hepatocytes apparently secreted *MDA*-7 processed protein(s) which, when administered to either Ad.*mda*-7 infected (FIGURE 15B) or AS K-*ras* oligonucleotide transfected (FIGURE 16B) MIA PaCa-2 pancreatic carcinoma cells, suppressed cell growth. Moreover, when assayed 84 hours post-infection, biological activity (as evidenced by growth suppression) was also observed when Ad.*mda*-7, but not Ad.*vec*, infected hepatocyte lysates were added to sensitized pancreatic carcinoma cells (FIGURES 15A and 16A). These results indicate that infection with Ad.*mda*-7 resulted in the production of secreted *MDA*-7 protein, and that this protein can affect appropriately sensitized pancreatic cancer cells when administered via the surrounding medium.

The biological effect appeared to be greatest on MIA PaCa-2 cells treated with AS K-*ras* oligonucleotides (FIGURES 16A and B). A significant growth inhibition was also observed in Ad.*mda*-7 infected MIA PaCa-2 cells (FIGURES 15A and B), suggesting possible synergy between intracellular pathways and membrane-mediated signal transduction pathways. In contrast, no significant effect was apparent

when untreated MIA PaCa-2 cells were exposed to either the infected hepatocyte lysate or culture supernatant (FIGURES 14A and B).

In additional experiments, it was also observed that Ad.*mda*-7 infected hepatocyte culture supernatant had a growth suppressive effect on cancer cells which are known to respond to increases in intracellular *MDA*-7 protein, in particular DU-145 human prostate cancer cells.

9. EXAMPLE: CO-INFECTION OF VIRAL VECTORS CARRYING *mda*-7 OR AS K-*ras* INHIBITS GROWTH OF PANCREATIC CANCER CELLS

9.1 MATERIALS AND METHODS

The following procedure was followed for four different pancreatic cancer cell lines, AsPC-1, BxPC-3, PANC-1, and MIA PaCa-2. Cells were seeded at 1 x 10^6/10 cm plate and the next day the cultures were either untreated (control) or infected with 100 pfu/cell of Ad.*mda*-7 or Ad.K-*ras* AS or 50 pfu/cell + 50 pfu/cell with Ad.*mda*-7 + Ad.K-*ras*AS (that is, infected with both viruses). After 2 hours of incubation with the various viruses, complete growth medium RPMI-1640 supplemented with 10 percent fetal bovine serum was added to the cells and they were incubated for an additional 6 to 8 hours at 37°C in a 5 percent CO_2/95 percent air humidified incubator. The cells were then resuspended and plated at 1 x 10^5/35 mm plate in triplicate and cells were incubated at 37°C under the same conditions. Two, four, six and eight days later the cell numbers were determined using a hemocytometer. Replicate samples varied by less than or equal to 10 percent.

9.2. RESULTS

The results are presented in FIGURES 17A-D. Infection with Ad.*mda*-7 alone (represented as a dashed line joining open circles in FIGURES 17A-D) had little effect on any of the cell lines tested. Infection with Ad.K-*ras* AS (represented as a broken line joining open triangles in the figures), which contains the 631 bp fragment of K-*ras* in an antisense orientation, as described above, inhibited the growth of the four pancreatic carcinoma cell lines to variable degrees; Ad.K-*ras* AS infection inhibited growth of MIA PaCa-2 (FIGURE 17D) and PANC-1 (FIGURE 17C) equally, had a smaller inhibitory effect on AsPC-1 (FIGURE 17A) and little

effect on BxPC-3 (FIGURE 17B). In contrast, co-infection of cultures with Ad.*mda*-7 and Ad.K-*ras* AS (represented as a dashed line joining squares with + overstrikes in the figures) had a synergistic inhibitory effect on the cell lines carrying an activating mutation in K-*ras* (AsPC-1, PANC-1 and MIA PaCa-2; FIGURES 17A,C and D), but no significant effect on BxPC-3, which has a wild-type K-*ras* gene (FIGURE 17B). These studies demonstrate that the combination of Ad.*mda*-7 + Ad.K-*ras* AS profoundly inhibited the growth of pancreatic carcinoma cells expressing a mutant K-*ras* gene without inducing this effect in wild-type K-*ras* carrying pancreatic carcinoma cells.

10. EXAMPLE: COMPARISON OF EFFECTS OF INFECTION WITH ADENOVIRUS VECTORS EXPRESSING *mda*-7 OR AS K-*ras* ALONE VERSUS A BIPARTITE ADENOVIRUS VECTOR CO-EXPRESSING *mda*-7 AND AS K-*ras* ON THE GROWTH OF PANCREATIC CANCER CELLS

10.1 MATERIALS AND METHODS

Cell culture and viability assays. The AsPC-1, MIA PaCa-2, PANC-1 and BxPC-3 human pancreatic carcinoma cell lines were cultured in RPMI medium containing 10% FBS at 37° C in a 95% air, 5% CO_2 humidified incubator. Cell growth and viability was monitored by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) staining technique as described in (Lebedeva et al., 2000, Cancer Res. 60:6052-6060).

Adenovirus infection protocol. Different replication incompetent adenoviruses were constructed which include Ad.*mda*-7 (FIGURE 2), Ad.AS.K-*ras* (FIGURE 3) and Ad.*bpv* (FIGURE 4). An empty adenoviral vector (Ad.*vec*) was used as a control. For MTT assay, 1500 cells were plated in each well of a 96-well plate. The next day, infection with adenovirus was carried out at a multiplicity of infection (MOI) of 100 pfu/cell. The cells were incubated with virus in 30 µl of serum-free medium for 2 hr to allow the complete adsorption of the virus. After 2 hr the cells were cultured in complete growth medium. For other experiments, $1x10^6$ cells were plated in a 10-cm dish. Adenovirus infection was carried out the next day in 1.5 ml of serum-free medium for 2 hr and then the medium was replaced with complete growth medium. Cell viability was assessed by MTT assay at day 1, 3, 5 and 7 post-infection.

Total RNA extraction and Northern blot analysis. Cells were infected with the adenovirus vectors at a multiplicity of infection (MOI) of 100pfu/cell. Expression of *mda*-7 was analyzed by Northern Blot analysis using a ^{32}P-dCTP labeled *mda*-7 cDNA probe. At two days post-infection, total RNA was extracted by Qiagen RNeasy mini kit according to the manufacturer's protocol. 5 μg of total RNA was denatured at 70°C for 10 min, electrophoresed in a 1% agarose gel containing formaldehyde and transferred to a nylon membrane. The membrane was hybridized with ^{32}P-dCTP labeled *mda*-7 cDNA probe using ExpressHyb hybridization solution (Clontech, Palo Alto, CA) according to the manufacturer's protocol. Following hybridization the membrane was washed and exposed for autoradiography.

Preparation of whole cell lysate and Western blot analysis. Cells were either uninfected (control) or infected with either Ad.*vec* or Ad.*bpv* at a multiplicity of infection (MOI) of 100 pfu/cell. At three days post-infection, cells from a 10-cm dish were harvested in 900μl RIPA buffer [1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS in phosphate-buffered saline (PBS) with protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN)]. The DNA was sheared by passing through a 21-gauge needle and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was used as total cell lysate. Thirty micrograms of total cell lysate from each sample were run in a 12.5% SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane using an electroblotting apparatus. The membranes were blocked with Blotto A [10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 5% skimmed milk and 0.05% Tween-20] for 1 h at room temperature and incubated with antibodies against MDA-7 (1:1000; rabbit polyclonal) and K-RAS (1:200; mouse monoclonal) at 4°C overnight. The membranes were washed three times for 5 min each with TBS-T [10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl and 0.05% Tween-20] and incubated with horseradish peroxidase conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:5000) in Blotto A for 1 h at room temperature. The membranes were washed in TBS-T 10 min each for 3 times. Chemiluminescence was detected by ECL western blotting detection kit (Amersham International plc, Buckinghamshire, UK) according to the manufacturer's protocol.

10.2. RESULTS

Ad.*bpv* was constructed for the purpose of expressing *mda*-7 and AS.K-*ras* from a single adenoviral vector. To confirm that infection with Ad.*bpv*

results in the production of *mda*-7 mRNA and protein, various pancreatic cancer cell lines were infected with either Ad.*vec* or Ad.*bpv* at a multiplicity of infection (MOI) of 100 pfu/cell. At 12, 24 and 48 hrs after infection, the cells were harvested and total RNA was extracted. The expression of *mda*-7 mRNA following Ad.*bpv* infection was analyzed by Northern blot analysis using ^{32}P-dCTP labeled *mda*-7 cDNA probe. As shown in FIGURE 18, *mda*-7 mRNA could not be detected in the cells infected with Ad.*vec*. However, a high level of *mda*-7 mRNA could be detected in the cells infected with Ad.*bpv*. This finding indicated that Ad.*bpv* infection leads to the production of *mda*-7 mRNA.

To confirm that the *mda*-7 mRNA that is transcribed following Ad.*bpv* infection can be efficiently translated into MDA-7 protein, Mia Paca-2 cells were infected with Ad.*vec* or Ad.*bpv* at a MOI of 100 pfu/cell. Cells were harvested after 3 days and total cell lysates were produced. The expression of MDA-7 and K-RAS proteins was detected by Western blot analysis using anti-MDA-7 and anti-K-RAS antibodies. As shown in FIGURE 19, MDA-7 protein could not be detected in the control (uninfected) and Ad.*vec* infected cells. However, MDA-7 protein could be detected in Ad.*bpv* infected cells. The expression of K-RAS protein could be detected in the control and Ad.*vec* infected cells, but could not be detected in cells infected by Ad.*bpv* infected cells (data not shown). These results confirm the hypothesis that Ad.*bpv* infection would result in the efficient production of MDA-7 protein and downregulation of K-RAS protein.

The effect of Ad.*bpv* infection on the growth of pancreatic cancer cell lines was next tested. Mutant K-*ras* containing pancreatic cancer cell lines Panc-1, Mia Paca-2 and AsPc-3 and wild type K-*ras* containing cell line BxPc-3 were infected with Ad.*vec*, Ad.*mda*-7, Ad.AS.K-*ras*, combination of Ad.*mda*-7 and Ad.AS.K-*ras* and Ad.*bpv*. The infections were carried out at a MOI of 100 pfu/cell. The growth of the cells was monitored on day 1, 3, 5 and 7 post-infection by MTT assay. As shown in FIGURE 20A-D, control (uninfected), Ad.*vec* and Ad.*mda*-7 infected cells continued to grow at a similar rate in all cell lines. Infection with Ad.AS.K-*ras* alone resulted in about 20% reduction in cell number in Mut K-*ras* containing cell lines. The combination of Ad.*mda*-7 and Ad.AS.K-*ras* resulted in the complete inhibition of cell growth in Mut K-*ras* containing cell lines. Infection with Ad.*bpv* alone was as potent as the combination in inhibiting the growth of Mut K-*ras* containing cell lines. None of the infection protocols could inhibit the growth of the wild-type K-*ras*-

containing BxPc-3 cells. These results indicate that Ad.*bpv* can be effectively used instead of the combination of Ad.*mda*-7 and Ad.AS.K-*ras* in inhibiting pancreatic cancer cell proliferation. The use of a single adenovirus vector in place of a combination of two adenovirus vectors is advantageous because it considerably reduces the potential toxicity associated with infection. Thus, Ad.*bpv* should be an efficacious tool for therapeutic purposes of pancreatic carcinoma.

Various publications and GenBank Database sequences are cited herein, the contents of which are incorporated by reference in their entireties.